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The Journal of Biochemistry, Vol. 44, No. 5, 1957

STUDIES ON THE METABOLISM OF UREA AND OTHER NITROGENOUS ~~COMPOUNDS~~ IN CHLORELLA ELLIPSOIDEA

I. ASSIMILATION OF UREA AND OTHER NITROGENOUS COMPOUNDS BY NITROGEN-STARVED CELLS*

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(Received for publication, November 16, 1956)

It has been reported by Tamiya *et al.* (*1*) and also by Walker (*2*) that *Chlorella* utilizes urea as the sole source of nitrogen, although no appreciable activity of urease is detected in this alga. This fact indicates that the mechanism of assimilation of urea in this alga might differ from that in higher plants in which urea is believed to be assimilated *via* ammonia, the first step of the process being the hydrolysis of urea by the action of urease (*3*, *4*, *5*, *6*).

The present work was undertaken with the view to get information as to the mechanism of presumably urease-free assimilation of urea by the green alga. Working with *Chlorella vulgaris*, Arnow *et al.* (*7*) have shown that besides urea, various nitrogenous compounds such as arginine, citrulline and ornithine could also be effectively utilized by the organism as the sole source of nitrogen. Considering the fact that these amino acids are the intermediates participating in cyclic mechanism of urea synthesis in mammalian tissues, the mode of utilization of these amino acids was also studied in parallel. It was revealed that the nitrogen sources studied were all rapidly assimilated by algal cells which had been subjected previously to N-deficiency, and that the assimilation was accompanied by a marked increase of endogenous respiration with simultaneous decrease of carbohydrate reserve in cells, indicating that the assimilation process demands the expenditure of respiratory energy (*8*, *9*). Detailed studies were, therefore, made on the correlation between the N-assimilation and endogenous respiration.

METHODS

Culture of Alga—The organism used was *Chlorella ellipsoidea* which was grown semiheterotrophically using a mineral-glucose-medium under illumination with daylight fluorescent lamps. Culture chambers used were oblong flat flasks deviced by Tamiya *et al.* (*10*). During the culture, air was constantly bubbled through the cell suspension. The culture temperature was 22°. The medium used for growing nitrogen-starved cells had the following composition: KH_2PO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $FeSO_4 \cdot 7H_2O$, 0.003 g; glucose, 20 g; Arnon's A5 solution (*11*), 1 ml. per liter. After 7 to 10 days of culture, the cells were collected by centrifugation, washed twice with distilled water and then used for further experiments. As a control normal cells were grown by adding urea (3 g. per liter) to the above-mentioned medium. As will be shown later, N-starved cells showed, as compared with normal cells, lower CQ-values (*12*), indicating that they are in a state more reduced than normal cells.

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* This work was announced at the Annual Meeting of the Botanical Society of Japan held in Hiroshima on Oct. 13, 1955.

Measurement of N-assimilation—Assimilation of N-compounds was followed by suspending N-starved cells in the media containing 4–8 millimoles per liter of N-compounds to be tested, besides non-nitrogenous mineral nutrients: KH_2PO_4, 1.25 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $FeSO_4 \cdot 7H_2O$, 0.003 g; Arnon's A5 solution (*11*), 1 ml., per liter. Ten ml. of the cell suspension was placed in a flask of 30 ml. capacity, and constantly shaken (with air in the gasphase) at 25° and in the dark. Aliquots were pipetted out at appropriate time intervals, and after centrifuging off the algal cells the quantity of N-compounds was measured by the following methods.

(*1*) *Ammonia:* Free ammonia was determined using Conway's microdiffusion units (*13*). One ml. of saturated solution of potassium carbonate was added to 0.5 ml. of the test solution. The units were left at 30° for 2 hours, by which time the ammonia was practically completely transfered to the standardized acid solution which was then titrated.

(*2*) *Urea:* Urea was estimated as ammonia (see above) after its enzymatic conversion by urease (13). Urease was prepared from soy bean seeds by Jakoby & Sugga's method (*14*). One ml. of urease solution was added to 0.5 ml. of the solution to be assayed, and incubated at 30° for 40 minutes to complete the hydrolysis of urea. Knivett's colorimetric method (*15*) for the estimation of urea using diacetylmonoxime was also adopted.

(*3*) *Arginine, Citrulline, and Ornithine:* The amount of arginine was measured by Sakaguchi's method (*16*). Citrulline was determined by the diacetyl method after Fearon modified by Knivett (*15*) with the use of arsenic oxide-hydrochloric acid as oxidizing agent. Ornithine was determined by Chinard's method (*17*) using ninhydrin and glacial acetic acid. In all these estimations optical density was determined at appropriate wave lengths with a Beckman spectrophotometer Model DU, or with a simpler colorimeter provided with a selenium photovoltaic cell. The wavelengths for determination of arginine, citrulline, ornithine and urea were 480–530, 492, 515, and 480 mμ, respectively.

(*4*) *Nitrate and Nitrite:* The amounts of nitrate plus nitrite were estimated as ammonia after their conversion into ammonia by reduction with Devarda's alloy (*13*). Nitrate content was separately determined as ammonia after vaporization of nitrite by the addition of hydrochloric acid to the mixture. The quantity of nitrite was determined as the difference between the above two readings.

Measurement of Respiration—In parallel with the above experiments, the respiration of N-assimilating cells (at 25° and in the dark) was followed by the Warburg technique. Each vessel contained 2.0 ml. of cell suspension and 0.5 ml. of test solution containing 20–40 millimoles per liter of N-compound besides the non-nitrogenous minerals (see above). Gas phase was air and the pH of solution was adjusted to 5.5 with dilute KOH. Determination of oxygen uptake was done by placing 0.5 ml. of 10 per cent KOH in the center well. Output of carbon dioxide was measured by parallel runs with and without KOH solution in the center well.

Measurement of Carbohydrate Reserve in Cells—During the course of N-assimilation more or less marked decrease of the carbohydrate reserve in algal cells was observed, which may be attributed to their consumption as the substrate of respiration. With the samples used for determining the assimilation of N-compounds, the content of carbohydrates in cells was measured by the method according to Syrett (*8*). After centrifuging off the solution, the cells were heated for 5 minutes in a boiling water bath, and then suspended in 5 ml. of 70 per cent (*v*/*v*) ethanol and kept at 30° for 3 days. The cells and extract were separated by centrifugation, and the former were subjected again to the extraction with 70 per cent ethanol. The procedure of extraction was repeated three times. Using the extracts thus obtained, reducing sugar was determined by the Hagedorn-Jensen method (*18*), and the non-reducing sugar was estimated from the increase in reducing capacity after hydrolyzing the extract with 0.5 *N* hydrochloric acid for 10 minutes at 100°. On the other hand, the cell residue was subjected to hydrolysis for 3 hours at 100° with 3 per cent sulfuric acid, followed by neutralization with potassium hydroxide, and by measuring the reducing capacity of the final solution the amount of insoluble carbohydrate was determined. The amounts of all carbohydrates were expressed in terms of glucose-equivalent.

RESULTS

The Course of N-Assimilation by N-Starved Cells—Using ammonium sulfate, potassium nitrate, urea, arginine, ornithine and citrulline as N-sources, the course of their assimilation (in the dark and under aerobic condition) by N-starved cells were followed by measuring the residual amount of the N-compounds in the medium. In all experiments the initial concentration of the N-compounds supplied was 0.004–0.008 mole per litre. The results obtained are reproduced by Curves A in Figs. 1–6, from which it may be seen that the order of rapidity of assimilation (at least under the condition applied in the experiment) was ; ammonium $>$ arginine $>$ urea $>$ nitrate $>$ ornithine $>$ citrulline. Using the most rapidly assimilated N-compounds, ammonium salt and arginine, the effect of their renewed provision after the complete consumption of their first administration was investigated. As is apparent from Curves A in

Figs. 7 and 8, the assimilation of N-sources supplied subsequently were more or less slower than it was the case with the first administration. The complete or almost complete consumption of the successively supplied N-sources shows, however, that the demand for N-sources of the N-starved cells had not yet been satisfied by the repeated supply of the substances. Two additional facts were revealed by this series of experiments: (1) Analysis of ammonium in the medium as well as in algal cells showed that in the course of assimilation of arginine, urea, nitrate, ornithine and citrulline, no trace of ammonium could be detected as an intermediate or a byproduct of N-metabolism, and (2) in the course of assimilation of nitrate, production of nitrite was detected at a certain stage (See Fig. 6., A). It is interesting to note that the maximum amount of nitrite was found at the stage at which the rate of nitrate

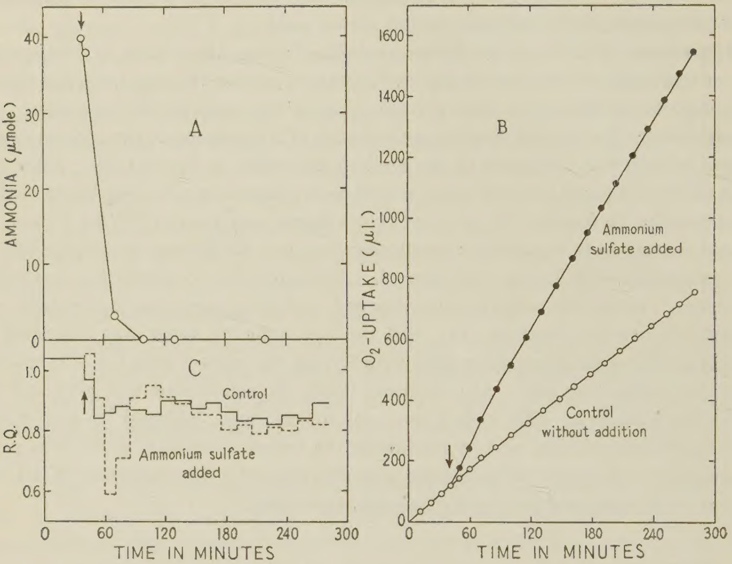

FIG. 1. Assimilation of ammonia by N-starved cells and its correlation with the respiration. Curve A: Assimilation of ammonia; Curve B: Effect of ammonia on the O_2-uptake; Curve C: Effect of ammonia on the respiratory quotient. Test volume: 10 ml. in A, and 2.5 ml. in B and C. 2 μ moles ammonium sulfate per ml. were added at the time indicated by arrow. The suspension contained 20 mg. of cells (dry weight) per ml.

assimilation was at its minimum.

Modification of Cell Components as a Result of N-Starvation and N-Assimilation—It has been shown by Spoehr and Milner (*19*) that the lipid content of *Chlorella* cells increased considerably when the cells were grown in N-deficient medium. The change of cell composition was manifest in the change of the combustion quotient (CQ) of cell material, which is a value proposed by Tamiya (*12*) to express the oxidoreductive state of organic materials as a whole. This quotient is calculated by the formula :

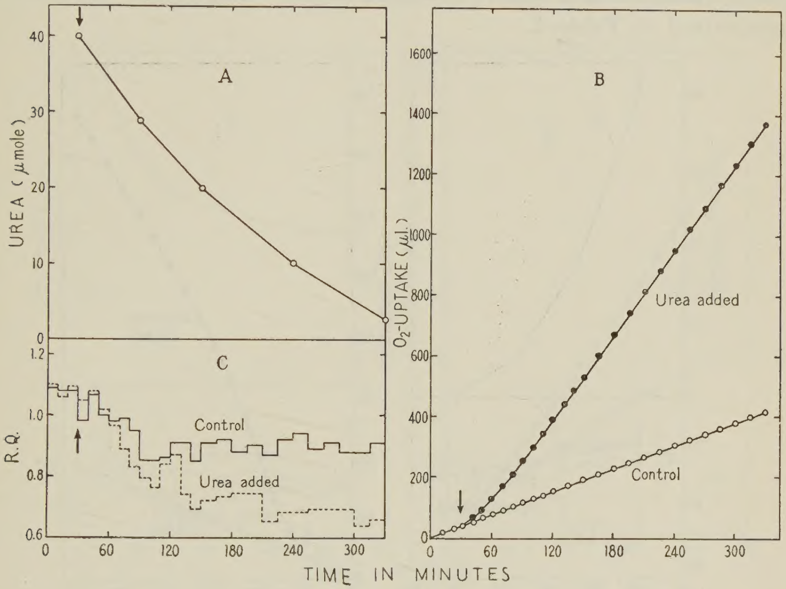

FIG. 2. Assimilation of urea by N-starved cells and its correlation with the respiration. Curves in A, B and C represent, respectively, the corresponding one with those in Fig. 1. (4 μ moles urea and 19 mg. of cells (dry weight) per ml.).

$$CQ=\frac{4n_C}{4n_C+n_H-2n_O-3n_N}$$

where n_C, n_H, n_O and n_N are relative numbers of carbon, hydrogen, oxygen and nitrogen atoms, respectively, contained in the material. The value $CQ=1$ indicates the oxidoreductive state equivalent to that of carbohydrate, and the values $CQ>1$ and $CQ<1$ signify the states that are more oxidized and more reduced, respectively, than the carbohydrate level. In accordance with the observation by Spoehr and Milner, the CQ-value of N-starved cells was found to be remarkably smaller than that of normal cells (grown with sufficient provision of urea). This fact indicates that the recovery in question involves an oxidative process, as will be discussed below. Using ammonium sulfate, urea and arginine, we investigated in what manner the elementary composition of N-starved cells be modified as a result of N-assimilation. The results obtained are summarized in Table I.

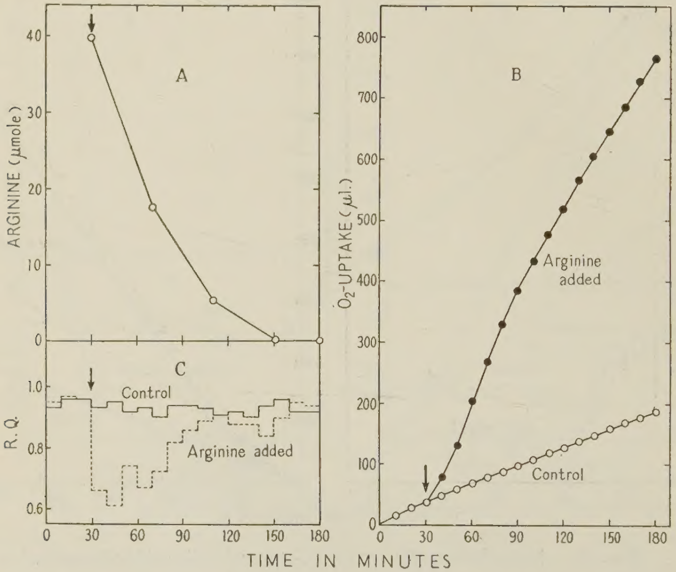

FIG. 3. Assimilation of arginine by N-starved cells and its correlation with the respiration. Curves in A, B and C represent, respectively, the corresponding one with those in Fig. 1. (4 μ moles L-arginine and 19 mg. of cells (dry weight) per ml.).

The fact that the N-content of cells after consumption of N-compound did not attain the cerresponding levels of normal cells indicates again that the demand of cells for N-sources was not fully satisfied by the quantity of N-compound applied. There was no significant change in CQ-value beyond the limits of experimental error before and after the incubation.

Correlation between Respiration and N-Assimilation—When N-compounds were supplied to N-starved cells, a marked increase in O_2-uptake was observed without exception. The phenomenon is unequivocally shown by the results given in Figs. 1–6, B. All these experiments were performed in parallel (under the same condition) with the above stated experiments shown in Figs. 1–6, A. Noteworthy is the fact that the enhanced respiration caused by the addition of N-compounds lasted even after the complete consumption of the added N-compounds. As shown by the results given in Figs. 7–8, renewed N-supply after the total consumption of N-sources in the medium caused only a slight increase in O_2-uptake which had already been enhanced by the preceeding N-supply. These results suggest that (1) the enhancement of respiration observed is related not only to the process of intake of N-compounds but also to the process of their further assimilation into cell material, and that (2) the enhanced O_2-uptake is not due to the respiration using the added N-compounds as substrates.

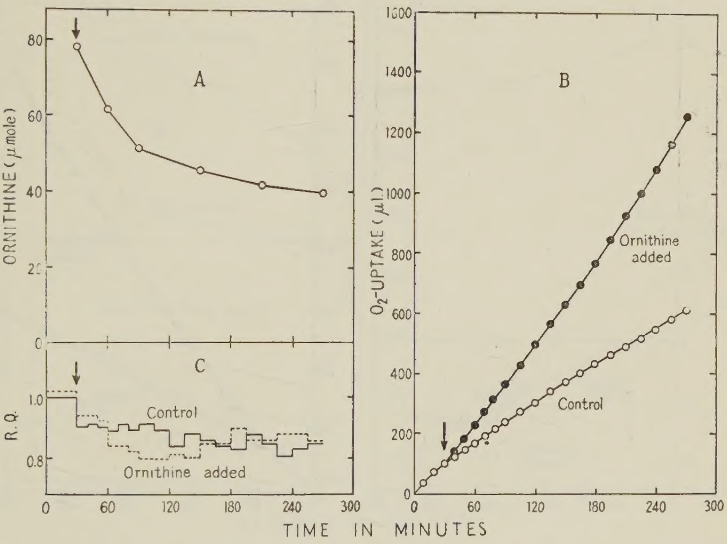

FIG. 4. Assimilation of ornithine by N-starved cells and its correlation with the respiration. Curves in A, B and C represent, resepctively, the corresponding one with those in Fig. 1 (8 μ moles DL-ornithine and 21 mg. of cells (dry weight) per ml.).

These results are essentially in conformity with the findings reported by Syrett (*8*), with *Chlorella vulgaris* and with ammonia. The different behaviour of Syrett's organism, in which the enhanced respiration decreased to almost the original level as soon as the added ammonia disappeared from the reaction medium, is most probably due to the difference in duration of starving the algal cells before use. In fact, Syrett treated the harvested cell in the mineral solution for 16–20 hours (at 25° under stationary bubbling with 0.5 per cent CO_2) while we prolonged the treatment for 7–10 days (at 22°, in the glucose-mineral medium), the final N-content of resulting starved cells being 4.8–5.8 per cent and 1.8–2.5 per cent in his and in ours, respectively.

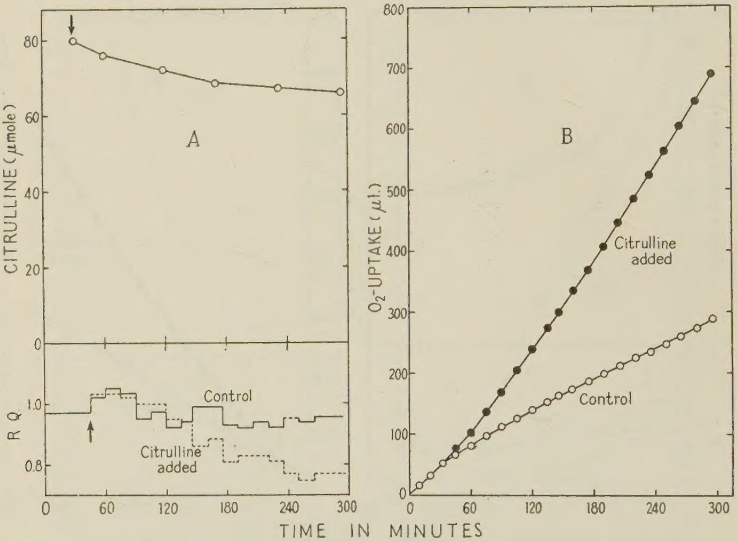

Fig. 5. Assimilation of citrulline by N-starved cells and its correlation with the respiration. Curves in A, B and C represent, respectively, the corresponding one with those in Fig. 1. (8 μ moles DL-citrulline and 17 mg. of cells (dry weight) per ml.).

That the process of N-assimilation is closely linked with respiration is also evident from the fact that the N-assimilation is completely, or almost completely, halted in the absence of oxygen (see the results shown in Fig. 9). To make clear whether the enhanced O_2-uptake accompanying the N-assimilation is due to the oxidation of endogenous respiration substrate, the quantity of carbohydrate contained in cells was analysed before and after the N-assimilation (N-compound tested being ammonium sulfate, urea and arginine). The results given in Table II show clearly that the quantity of intra-cellular carbohydrate decreased markedly as a result of N-assimilation. We may, therefore, infer that carbohydrate was the principal, if not the sole, substrate in the enhanced endogenous respiration observed. It may be remarked that the process of N-assimilation in question (when ammonium, urea or arginine was used as N-source) was not influenced appreciably by light, as it may be seen from the results given in Fig. 10.

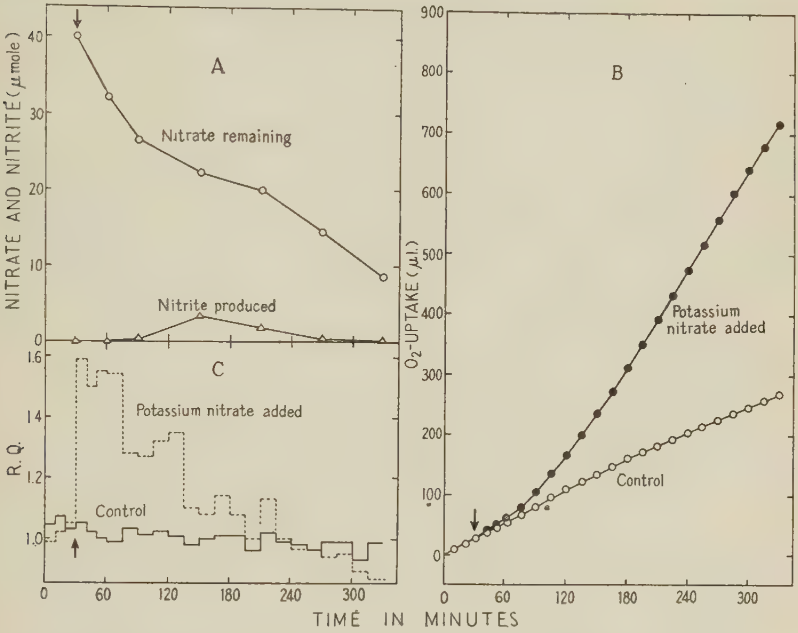

FIG. 6. Assimilation of nitrate by N-starved cells and its correlation with the respiration. Curves in A, B and C represent, respectively, the corresponding one with those in Fig. 1. (4 μ moles potassium nitrate and 10 mg. of cells (dry weight) per ml.).

Based on the data given in Figs. 1–6, the ratios,* N-compound assimilated (mole)/enhanced O_2-uptake (mole) and N-atoms assimilated/

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* The ratios were calculated using the values obtained during the first 30 minutes of experiments.

enhanced O_2-uptake (mole) were calculated. Simultaneous determinations of assimilated N-compounds and the enhanced consumption of O_2 and total endogenous carbohydrate were also made using ammonium sulfate, urea and arginine as N-sources. The results obtained are summarized in Table III and IV.

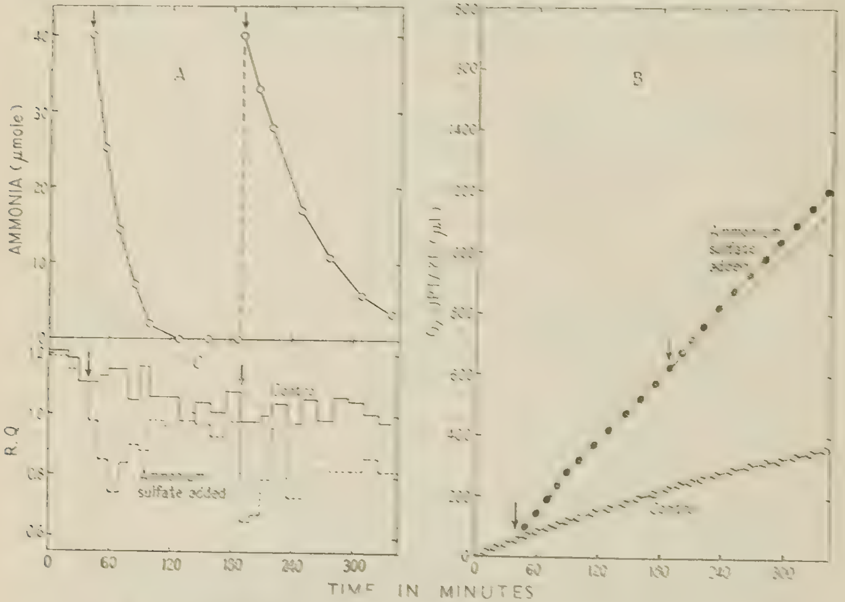

FIG. 7. Effect of successive additions of ammonia on its assimilation, the O_2-uptake and the respiratory quotient in N-starved cells. 2 μ moles ammonium sulfate per ml. were added successively at the times indicated by arrow. Curves in A, B and C represent, repectively, the corresponding one with those in Fig. 1. (The suspension contained 10 mg. of cells (dry weight) per ml. (after the first addition); Total volume: in A, 10 and 12 ml., respectively, after the first and second addition, and in B and C, 2.5 and 3.0 ml.).

Change of Respiratory Quotient During the Process of N-Assimilation—Manometric experiments performed in parallel with the experiments of determining the course of decrease of N-compounds in the medium (Figs. 1–6, A) showed that a marked change in the respiratory quotient (R.Q.) occurred during the process of N-assimilation. The relevant data are shown by Curves C in Figs. 1–6. In control experiments (*i.e.*

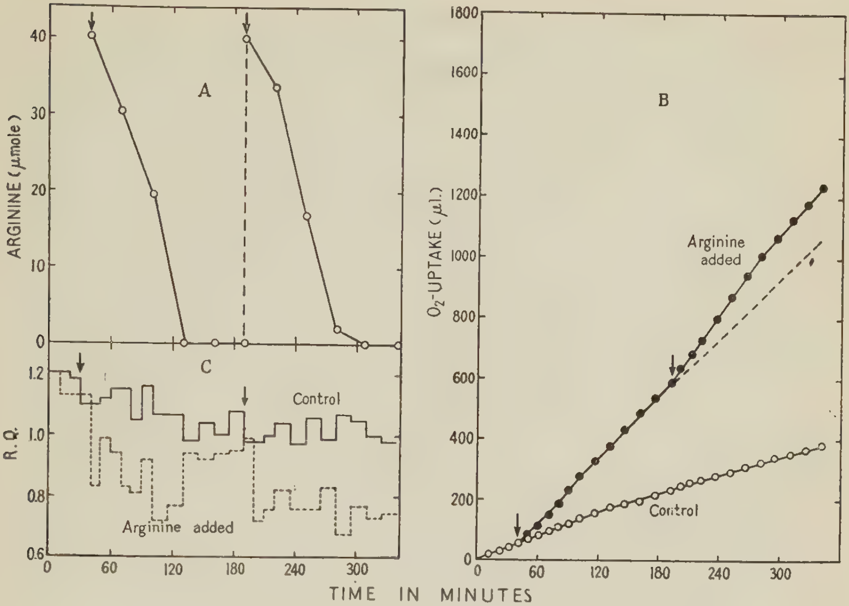

FIG. 8. Effect of successive addition of arginine on its assimilation, the O_2-uptake and the respiratory quotient in N-starved cells. Conditions of the measurement are the same as in Fig. 7., except for replacing ammonium sulfate with L-arginine.

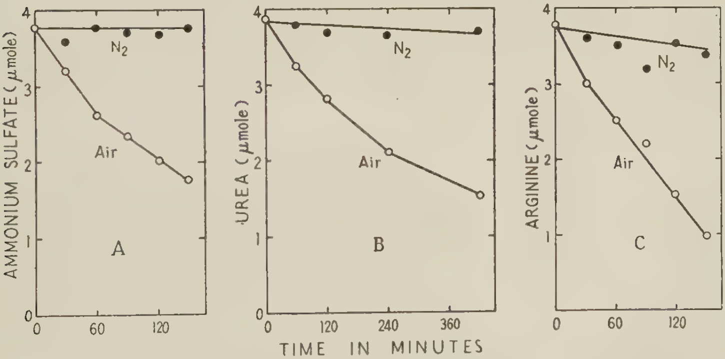

FIG. 9. Effect of anaerobiosis on N-assimilation by N-starved cells. (in A: 4 µ moles ammonium sulfate and 4 mg. of cells (dry weight); in B: 4 µ moles urea and 11 mg. cells; in C: 4 µ moles L-arginine and 4 mg. of cells, per ml.).

TABLE I

Elementary Composition of N-Starved Chlorella Cells before and after Incubation with Ammonium Sulfate, Urea and Arginine, and of Normal Cells

		N-starved cells					Normal cells
		before incubation	after incubation* with				
			ammonium sulfate	urea	arginine	none	
Content in per cent of dry substance	C	57.09	56.28	55.99	57.65	57.16	45.50
	H	8.03	7.98	7.91	8.26	8.06	6.89
	O	20.45	20.65	21.35	19.90	20.43	30.43
	N	1.83	2.40	2.06	2.42	1.85	7.96
	Ash	12.60	12.69	12.69	11.77	12.50	9.22
Combution quotient**		0.79	0.79	0.79	0.79	0.78	0.94

* Incubation times: 2 hours for ammonium sulfate and arginine and 5 hours for urea and the control without added N-compound. During the incubation period 3.7, 2.9 and 3.2 μ moles of ammonium sulfate, urea and arginine, respectively, were assimilated per 1 ml. of experimental suspension. 18 mg. of cells (dry weight) per ml., 25°, in the dark.

** The CQ-values were calculated on ash-free, dry weight basis.

without added N-compounds) lasting for several hours, the R.Q.-value either remained constant at about 0.9–1.05 or showed a gradual decrease from around 1.2 to 0.9. When nitrate was added as a N-source, a marked temporary increase in R.Q. (up to 1.6) was observed, a phenomenon which has been known since the classical work of Warburg and Negelein (*20*). Quite different phenomena were observed when other N-compounds were assimilated. In the case of ammonium sulfate, arginine and ornithine, a more or less abrupt temporary fall of R.Q. (down to 0.6–0.8) occurred, and in the case of urea and citrulline, R.Q. decreased progressively (down to 0.6–0.8) with the lapse of time. The phenomenon observed with the most rapidly assimilated N-compounds, ammonium sulfate and arginine, may be of special interest (Figs. 7–8, C). In these cases the R.Q.-value, which had decreased on the addition of N-compounds, increased again to the normal level when the N-compounds in the medium totally disappeared. When the N-compounds were added afresh at this stage, there occurred again the fall of R.Q. and its subsequent recovery to the normal level.

The increase in R.Q.-value occurring in the process of nitrate as-

TABLE II

Relation between the Assimilation of N-Compounds and the Consumption of Intracellular Carbohydrate

All values refer to 1.0 ml. of suspension. pH 5.6, 25°, in the dark. Carbohydrate given as μg. equivalent glucose; N-compounds as μg. nitrogen.

N-source	Time of incubation *hour*	Reducing sugar μg.	Non-reducing sugar μg.	Poly-saccharide μg.	Total intracellular carbohydrate μg.	N-compound μg.
Urea*	0	190	660	1690	2540	103
	4	170	480	1440	2090	45
	Difference	20	180	250	450	58
None*	0	190	670	1710	2570	—
	4	160	700	1660	2520	—
	Difference	30	-30	50	50	—
Ammonium sulfate**	0	115	99	415	620	93
	2	65	50	345	460	51
	Difference	50	49	70	169	42
Arginine**	0	128	124	430	688	168
	2	115	20	410	545	90
	Difference	13	104	26	143	78
None**	0	83	159	429	671	—
	2	79	165	412	658	—
	Difference	4	-6	15	13	—

* 7.3 mg. of cells (dry weight) per 1 ml. of suspension.

** 3.1 mg. of cells (dry weight) per 1 ml. of suspension.

similation may be explained, as it has already been discussed by other authors, as a result of reduction of nitrate, which is necessary for incorporation of nitrate-N into nitrogenous cell material. The general trend of decrease in R.Q., which was observed with other N-compounds, may require a special explanation. As mentioned already, N-starved cells show lower C.Q.-values, which are restored to normal C.Q.-values when the cells are supplied with sufficient amounts of N-sources. This process of recovery represents, as a whole, an oxidation process which can be realized, in the over-all balance sheet of cellular metabolism, only by the expenditure of O_2 in the environment. The fall of R.Q.-value in question may, therefore, be attributed to the occurrence of such an oxidative recovery process, and is by no means contradictory to our assumption that the principal substrate of the respiration coupling with

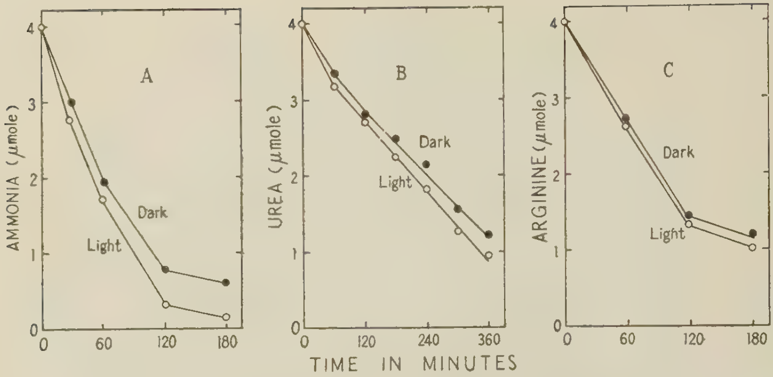

FIG. 10. Effect of light on N-assimilation by N-starved cells. The cell suspensions were illuminated from below with fluorescent lamp (light intensity: 3,000 Lux). In the control marked as " Dark ", flasks were covered with black polyvinyl sheets. (in A: 2 μ moles ammonium sulfate and 4 mg. of cells (dry weight); in B: 4 μ moles urea and 9 mg. cells; in C: 4μ moles L-arginine and 4 mg. cells, per ml.).

TABLE III

*Efficiency of N-Compounds Assimilation**

N-source	N-compound assimilated (mole) enhanced O_2-uptake (mole)	N-Atom enhanced O_2-uptake (mole)
Ammonium sulfate	0.7	1.4
Urea	1.01	1.0
Potassium nitrate	1.4	1.4
Arginine	0.83	3.3
Citrulline	1.08	3.2
Ornithine	1.58	3.2

* The ratios were calculated using the values obtained during the first 30 minutes of experiments presented in Figs. 1–6.

the N-assimilation is carbohydrate.

Effect of Various Poisons on the Rates of N-Assimilation and Respiration—To get further insight into the relationship between the process of N-assimilation and respiration, comparative studies were made on the effect of various poisonous substances on the rates of the two processes. N-

TABLE IV

Relation between Assimilation of N-compounds and Consumption of Intracellular Carbohydrate and Oxygen

The values in first three columns refer to 1 ml. of experimental suspension. 18 mg. of cells (dry weight) per ml., pH 5.6, 25°, in the dark. Incubation time: 2 hours for ammonium sulfate and arginine and 5 hours for urea. At zero time 4 μ moles of each compound was added. Carbohydrate given as μ moles equivalent glucose.

N-source	N-compound consumed μM	Total carbohydrate consumed μM	Oxygen consumed μM	Ratio $\Delta N : \Delta C\text{-}H : \Delta O_2$
Ammonium sulfate	3.6	3.7	11.6	
None	—	0.67	0.89	1.2 : 1 : 3.6
	ΔN 3.6	ΔC-H 3.0	ΔO_2 10.7	
Urea	3.5	4.9	23.0	
None	—	1.67	2.05	1.1 : 1 : 6.6
	ΔN 3.5	ΔC-H 3.2	ΔC-H 20.9	
Arginine	3.9	2.6	10.2	
None	—	1.67	0.89	2.1 : 1 : 4.9
	ΔN 3.9	ΔC-H 1.9	ΔO_2 9.3	

$$H_{(N)} = \frac{V^{\circ}_{N} - V_{N}}{V^{\circ}_{N}} \times 100$$

$$H_{(O_2)} = \frac{V^{\circ}_{O_2} - V_{O_2}}{V^{\circ}_{O_2}} \times 100,$$

where V°_{N} and $V^{\circ}_{O_2}$ are the rates of N-assimilation and enhanced O_2-uptake, respectively, in the absence of poison, and V_N and V_{O_2} the corresponding values in the presence of the poisons tested. For comparison, the effect of poisons on the background endogenous respiration (*i.e.* the respiration in the absence of added N-compound) was also measured. The degree of inhibition in this case was expressed by $H(\varepsilon)$. The concentration of poisons applied ranged from 4×10^{-5} to 4×10^{-3} mole per litre.

compounds used were ammonium sulfate and urea. Measuring the rate of consumption of N-compounds and the rate of enhanced O_2-uptake, both in the presence and absence of poisons, the degree of inhibition (H) was determined by the following relation:

Among the 19 poisons tested, the following substances showed no, or almost no inhibitory effect upon the process of N-assimilation and the enhanced O_2-uptake: arsenate, fluoride, urethane, thiourea, hydrazine, borate and chlorate. The strongest inhibition of both N-assimilation and enhanced O_2-uptake was brought by 2,4-dinitrophenol (DPN), azide and *p*-chloromercuribenzoate (PCMB). The results obtained with strong or moderately strong inhibitors are summarized in Table V. The following facts emerge from the data given in the table.

(1) Most of the inhibitors inhibited the N-assimilation and the enhanced O_2-uptake to almost the same degree (arsenite, azide, hydroxylamine, thiosemicarbazide, PCMB, chlorite, monofluoroacetate, monoiodoacetate and carbon monoxide).

(2) Some inhibitors, such as DNP, cyanide and monochloroacetate, affected the N-assimilation stronger than the enhanced O_2-uptake. There were no poisons which strongly inhibited the enhanced O_2-uptake without affecting the process of N-assimilation.

(3) Many substances exerted almost the same inhibitory effect upon urea- and ammonia-assimilation (DNP, azide, hydroxylamine, PCMB, chlorite, CO, and halogenated acetic acids). With thiosemicarbazide, however, the inhibition of urea-assimilation occurred more markedly than that of ammonia-assimilation, while the reverse was the case with cyanide and arsenite.

(4) A considerable difference was found between the enhanced O_2-uptake and the background respiration in their susceptibility towards inhibitors. Some substances, which strongly inhibited the enhanced O_2-uptake (and also N-assimilation), even showed an accelerating effect upon the background respiration (DNP, cyanide, azide, hydroxylamine, chlorite and carbon monoxide). With arsenite, PCMB, and halogenated acetic acids the inhibition of enhanced respiration was stronger than that of background respiration, while the reverse was the case with thiosemicarbazide.

The facts (1) and (2) indicate that the enhanced O_2-uptake is closely —and conceivably energetically—linked with the process of N-assimilation. Fact (3) suggests that the processes of urea- and ammonia-assimilation involve some common mechanism besides some different pathways. And finally, fact (4) implies that the mechanism of enhanced O_2-uptake is profoundly different from that of the background respiration.

The experimental data obtained with cyanide and DNP, are presented in some details in Figs. 11 and 12. From Fig. 11 it is apparent that the cyanide in the concentration 4×10^{-3} mole per litre, almost completely halted the ammonia-assimilation, while it was almost without effect upon the assimilation of urea. Curves in Fig. 12, in which

TABLE V

Effect of Various Poisons on the Rates of Ammonia and Urea Assimilation, and of Enhanced and Endogenous Respiration

4–12 mg. dry weight cells and 4 μ moles ammonium sulfate or urea in each 1 ml. of experimental suspension. pH 5.5, 25°, in the dark. $H_{(N)}$ was calculated from the values obtained during initial 2 hours and $H_{(O_2)}$ and $H_{(\varepsilon)}$ from those during initial 1 hour.

Nitrogen source		Ammonium sulfate			Urea		
Inhibitor	Conc. (*M/l*)	4×10^{-5}	4×10^{-4}	4×10^{-3}	4×10^{-5}	4×10^{-4}	4×10^{-3}
DNP	$H_{(N)}$	37	100	100	36	100	100
	$H_{(O_2)}$	0	100	100	0	100	100
	$H_{(\varepsilon)}$	- 58	- 92	100			
Arsenite	$H_{(N)}$	—	46	87	—	18	69
	$H_{(O_2)}$	—	87	100	—	64	89
	$H_{(\varepsilon)}$	—	48	58			
Cyanide	$H_{(N)}$	—	78	94	—	0	19
	$H_{(O_2)}$	—	45	70	—	0	0
	$H_{(\varepsilon)}$	—	- 54	- 94			
Azide	$H_{(N)}$	77	89	100	54	100	100
	$H_{(O_2)}$	79	100	—	75	100	—
	$H_{(\varepsilon)}$	-400	-120	—			
Hydroxylamine	$H_{(N)}$	—	25	76	—	26	100
	$H_{(O_2)}$	—	50	68	—	40	93
	$H_{(\varepsilon)}$	—	-130	-170			
Thiosemicarbazide	$H_{(N)}$	—	0	7	—	27	36
	$H_{(O_2)}$	—	—	0	—	—	26
	$H_{(\varepsilon)}$	—	—	35			
PCMB*	$H_{(N)}$	68	85	100	76	87	100
	$H_{(O_2)}$	58	100	—	40	100	—
	$H_{(\varepsilon)}$	17	56	—			
Chlorite	$H_{(N)}$	—	35	62	—	21	45
	$H_{(O_2)}$	—	—	56	—	—	32
	$H_{(\varepsilon)}$	—	—	- 78			
Monofluoroacetate	$H_{(N)}$	4	90	100	0	87	100
	$H_{(O_2)}$	—	81	100	—	52	100
	$H_{(\varepsilon)}$	—	- 37	- 27			

Nitrogen source		Ammonium sulfate			Urea		
Inhibitor / Conc. (M/l)		4×10^{-5}	4×10^{-4}	4×10^{-3}	4×10^{-5}	4×10^{-4}	4×10^{-3}
Monoiodoacetate	$H_{(N)}$	24	78	100	19	75	93
	$H_{(O_2)}$	21	100	—	31	100	—
	$H_{(\varepsilon)}$	- 25	47	—			
Monochloroacetate	$H_{(N)}$	—	32	53	—	28	65
	$H_{(O_2)}$	—	—	38	—	—	14
	$H_{(\varepsilon)}$	—	—	0			
		50%	70%	95%	50%	70%	95%
CO	$H_{(N)}$	42	78	90	30	75	100
	$H_{(O_2)}$	10	63	100	25	100	100
	$H_{(\varepsilon)}$	- 42	-111	- 25			

* Inhibition by PCMB was not restored with cystein treatment (1/150 M per litre).

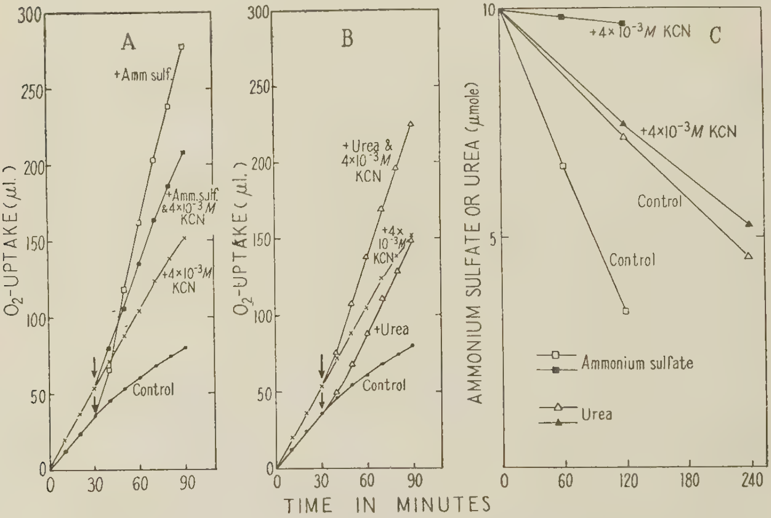

FIG. 11. Effect of cyanide on ammonia- and urea-assimilation by N-starved cells and on the corresponding enhanced O_2-uptake. (in A and B: 9.6 mg. cells and in C 10.8 mg. of cells (dry weight) per ml.; KCN: 4×10^{-3} mole/litre).

are plotted the degree of inhibition ($H_{(N)}$ and $H_{(O_2)}$ for urea and ammonia) *versus* logarithms of DNP-concentration, show that (1) the inhibitory effect of this poison is almost the same on both urea- and ammonia-assimilation, and that (2) in both cases N-assimilation was more strongly affected than the enhanced O_2-uptake. In this figure, also a curve showing the effect of DNP upon the background respiration is plotted. As may be seen, the background respiration was almost 100 per cent accelerated by 10^{-4} M DNP, although it was completely suppressed by higher concentrations (4×10^{-4} M) of the poison.

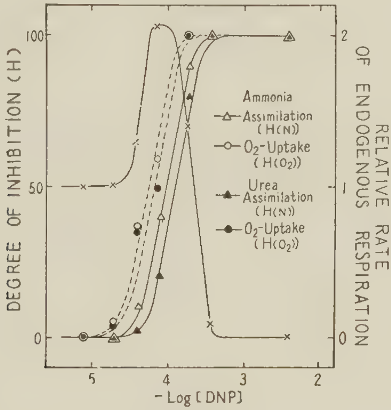

FIG. 12. Effect of 2,4-dinitrophenol on ammonia- and urea-assimilation by N-starved cells and on the corresponding enhanced O_2-uptake and the background respiration. (4 μ moles ammonium sulfate or urea; 4–10 mg. of cells (dry weight) per ml.). Open and solid circles: inhibition of ammonia- and urea-assimilation, open and solid triangles: inhibition of corresponding enhanced O_2-uptake, crosses: relative rate of endogenous background respiration.

Absence of Urease and Arginase in Algal Cells—Using various samples of algal cells grown in normal as well as in N-deficient media, the presence of urease and arginase was tested, but in no case positive results were obtained. The result of arginase test remained the same when Mn^{++} or Co^{++}, which are known to be effective in activating the enzyme, were added to the test solution. This fact, together with the observation described earlier that no trace of ammonia was detected in the medium when urea or arginine (and also when ornithine or citrulline) was assimilated by algal cells, seems to exclude positively the possibility that these N-compounds are assimilated *via* ammonia, as it is the case in many other organism.

SUMMARY

1. The assimilation of various nitrogenous compounds by the N-starved cells of *Chlorella ellipsoidea* was followed in connection with its respiratory metabolism.

2. Ammonia, nitrate, urea, arginine, citrulline and ornithine, added individually as the sole source of nitrogen, were utilized by the alga with the order of rapidity: ammonia > arginine > urea > nitrate > ornithine > citrulline.

3. No trace of ammonia was detected in the cell nor in the medium as an intermediary or byproduct of nitrogen metabolism during incubation with nitrate, urea, arginine, citrulline and ornithine. With nitrate, an intermediary and transient formation of nitrite was revealed.

4. The assimilation of N-compounds added to the medium was shown to be always accompanied by the enhanced respiratory metabolism, together with an increased consumption of cellular carbohydrate reserve.

5. The effects of anaerobiosis and various chemicals on the metabolic processes in question were investigated, and the results were discussed in regard to the possibility of the enhanced respiration furnishing energy necessary for the assimilation of added N-compounds.

6. No activity of urease nor of arginase was ever detected with the test organism studied.

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REFERENCES

(*1*) Tamiya, H., Hase, E., Shibata, K., Mituya, A., Iwamura, T., Nihei, T., and Sasa, T., Algal Culture from Laboratory to Pilot Plant, Ed. by John S. Burlew, Carnegie Inst. Wash. Pub., **600**, 204 (1953)

(*2*) Walker, J. B., *Proc. Natl. Acad. Sci.*, **38**, 561 (1952)

(*3*) Tauböck, K., *Oest. Bot. Z.*, **76**, 43 (1927)
(*4*) Pirschle, K., *Biochem. Z.*, **212**, 466 (1929)
(*5*) Yamaguchi, S., *J. Fac. Sci. Hokkaido Imp. Univ., Ser. V*, **4**, 47 (1935)
(*6*) Hinsvark, O. N., Wittwer, S. H., and Tukey, H. B., *Plant Physiol.*, **28**, 70 (1953)
(*7*) Arnow, P., Oleson, J. J., and Williams, J. H., *Am. J. Bot.*, **40**, 100 (1953)
(*8*) Syrett, P. J., *An. Bot., N. S.*, **17**, 1 (1953); *ibid.*, **17**, 21 (1953)
(*9*) Cramer, M., and Myers, J., *J. Gen. Physiol.*, **32**, 93 (1949)
(*10*) Tamiya, H., Iwamura, T., Shibata, K., Hase, E., and Nihei, T., *Biochim. et Biophys. Acta*, **12**, 23 (1953)
(*11*) Arnon, D. I., *Am. J. Bot.*, **25**, 322 (1938)
(*12*) Tamiya, H., *Acta Phytochem.*, **6**, 227 (1932); Advances in Enzymology, Interscience Pub., Inc., N. Y., **2**, 183 (1942)
(*13*) Conway, E. J., Microdiffusion Analysis and Volumetric Error, 3rd. Ed., Crosby Lockwood & Son, London, (1950)
(*14*) Jakoby, M., and Sugga, *Biochem. Z.*, **69**, 116 (1914)
(*15*) Knivett, V. A., *Biochem. J.*, **56**, 606 (1954)
(*16*) Sakaguchi, K., *J. Biochem.*, **37**, 231 (1950); *ibid.*, **38**, 91 (1951)
(*17*) Chinard, F. P., *J. Biol. Chem.*, **199**, 91 (1952)
(*18*) Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923)
(*19*) Spoehr, H. A., and Milner, H. W., *Plant Physiol.*, **24**, 120 (1949)
(*20*) Warburg, O., and Negelein, E., *Biochem. Z.*, **110**, 66 (1920)

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STUDIES ON PROTAMINES

I. AMINO ACID COMPOSITION AND HOMOGENEITY OF CLUPEINE, SALMINE AND IRIDINE

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Protamines consist of comparatively few kinds of amino acids and have smaller molecular weight. They belong to a group of special proteins which mainly consist of basic amino acids, especially of arginine. The protamines, therefore, seemed to be a good material for the investigation of protein structure.

Many studies, indeed, have been made in this field (*1*, *2*, *3*). It was, however, not until recent years that light was thrown upon the chemical structure of protamines based on confirmed experimental evdiences.

The first thing we should undertake is to study the amino acid composition, molecular size and amino acid sequence in the molecules. Since many new methods such as microbioassay and chromatography have recently been introduced in the determination of amino acids, it is a matter of course to reinvestigate the composition of protamines. Because of their low molecular weight, its determination by an ultracentrifugal method is difficult, thus it should be done by some other appropriate methods. As for the amino acid sequence in the molecules, various recently developed methods have indeed been introduced in the field of protamine studies.

From such a point of view, some of the present authors have carefully reinvestigated in the last several years on some protamines: salmine, iridine and especially clupeine. They have discovered some new facts, of which brief reports have already been published a few years ago (*4*, *5*). The details of the experiments and also the results obtained later will now be reported successively in a series of papers. The present publication deals with the preparation, the qualitative amino acid composition and the problem of the homogeneity of the protamines.

RESULTS AND DISCUSSION

I. The Preparation of Protamines

Fresh and mature milts or sperm of herring (*Clupea pallasii*) and salmon (*Oncorhynchus keta*) from Hokkaido, the north end of Japan, and rainbow-trout (*Salmo irideus*) from Okutama near Tokyo, were used as material. Clupeine was prepared from herring, salmine from salmon and iridine from rainbow-trout. The method of preparation is as follows. The material was extracted with cold dilute hydrochloric acid. Protamines thus obtained were made into picrates, and were fractionated tentatively into two parts, making use of their solubility difference in aqueous acetone. These two fractions, the more and the less soluble ones, were then transformed respectively into sulfates. Protamine sulfates thus obtained formed colorless glittering substances.

The above treatment was mostly similar to the Rasmussen's procedure (*6*), which so far seems to be the best. In the treatment the material was exposed for a limited time to dilute acid at low temperature. Thus, it was intended to prevent any change in protamine itself or contamination with basic components which might occur due to decomposition of nucleic acid.

The samples of protamine sulfates showed their proper absorption spectra (Fig. 1), and no absorption band in the region of wave length longer than 250 mμ. They were thus confirmed to contain no appreciable amount of nucleic acid or its components.

A milder method of preparation was further desired for fear that protamine might suffer any change in its nature during the above procedure. For this purpose a method was attempted, in which an old method of extracting protamine with cupric chloride by Kossel (*1*) was combined with a recent one of extracting nucleoprotamine with a saline solution by Mirsky (*7*). Specimens of protamines thus prepared without acid treatment were also examined on some properties. However, the results described below are limited to those obtained with samples prepared through the usual acid extraction.

II. Qualitative Amino Acid Composition of Protamines

The results of paper chromatographic studies on relative content of amino acids in some fractions of protamines are shown in Table I.

In the table it is shown that there are some differences in the components when compared with the results of protamines reported by

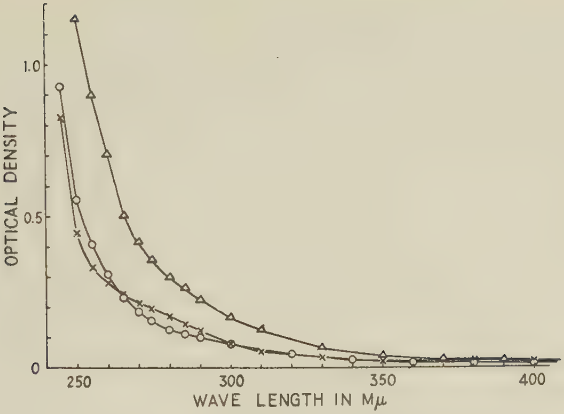

FIG. 1. The absorption spectra of some specimens of protamine sulfates.

Read on a Beckman quartz spectrophotometer, Model DU, with the same optical path-length, 1 cm.

○—○ Clupeine sulfate, less soluble (0.98_4 per cent aqueous solution, pH 2.6_3, 29–32°)

△—△ Clupeine sulfate, more soluble (1.01 per cent aqueous solution, pH 2.9_8, 31–33°)

×—× Salmine sulfate, less soluble (1.11 per cent aqueous solution, pH 2.7_1, 29–32°)

TABLE I

*Relative Content of Amino Acids in Protamines**

Clupeine (Less sol.**, 1947***)	Arg≫Ala≥Ser>Val>Thr>Ileu≥Gly; Pro
Clupeine (More sol., 1947)	Arg≫Ala>Ser>Ileu≥Thr>Val≥Gly; Pro
Salmine (Less sol., 1950)	Arg≫Ser>Val≥Gly>Ala≥Ileu; Pro
Iridine (1952)	Arg≫Gly, Ser, Val>Ala>Ileu; Pro

* Observed on one- and two-dimensional paper chromatograms. The amount of proline cannot be compared on a paper with those of other constitutents on account of its yellow coloration with ninhydrin.

** The protamine obtained from the less soluble picrate in aqueous acetone.

*** The year in which the material fish was obtained.

foreign authors. Although glycine as a component has never been reported in specimens of clupeine from any laboratory (*8–12*) except English and Czechoslovak ones (*13*, *14*), it was now discovered either in the less or the more soluble fraction of our clupeine*. Indeed, it was shown that glycine was contained in the same amount as isoleucine in the former fraction by the result of its quantitative analysis, which will be reported later. As to the other constituent amino acids no difference was found.

Isoleucine has either been reported to be present (*10*, *15–19*) or not (*8*, *13*, *20*) as a component of the specimens of salmine by several researchers, while it was shown to be present in our specimen.

The amino acid composition of our specimen of iridine was quite similar to that of our salmine. Lysine was not found, and hence our result differed from that of Felix (*21*).

All these disagreements reported in the composition of each protamine may partly be due to the species specificity.

The results of accurate quantitative determination by ion-exchange chromatography of amino acid composition of a less soluble specimen of both clupeine and salmine will be described in detail in a following paper.

III. Homogeneity of Protamine

It is a question to be elucidated whether our protamines are homogeneous or not.

Specimens of clupeine and salmine similarly prepared, though not fractionated, from the same material in Dr. Watanabe's laboratory in this institute were shown to be almost homogeneous in view of the results of diffusion (*22*), electrophoresis (*23*) and sedimentation (*24*). It was also confirmed by the diffusion measurement (*22*) that a specimen of our less soluble clupeine was homogeneous.

From the result on clupeine as shown in Table I, a difference in relative content of amino acids between the less and the more soluble fractions was found, as indicated in a reversal of the order of relative content of valine and isoleucine. This fact appeared to agree with the recent result by Waldschmidt-Leitz (*25*) on clupeine. It seems probable from the above that there was some inhomogeneity in the specimen of clupeine isolated in our laboratory. Another specimen was also fractionated into four parts under a slightly modified condition and the relative content of amino acids in each part was examined by paper chromatography (Table II). The table also shows some inhomogeneity in this specimen. The content of glycine especially varied in this case. There is, however, some doubt regarding the content of glycine in this case, since the absence of nucleic acid has not yet been confirmed.

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* Although glycine was shown to occur in an acid hydrolysate of nucleic acid (*33*), the absence of the latter in the present specimens was confirmed by the absorption spectra.

An application of counter-current distribution to a specimen of clupeine sulfate also indicated some inhomogeneity (Fig. 2). Felix has also pointed out a similar fact (*26*). In our laboratory the distribution was made between an aqueous solution of sodium *p*-toluene sulfonate containing sodium chloride and *n*-butanol. The content of clupeine in each tube was determined by retention analysis, using a solution of an acid dye, Ponceau 6RF, as a developer (see experimental part).

TABLE II*

*Relative Content of Amino Acids in Four Tentative Fractions of a Clupeine***

Fraction	1	Less sol.	Arg⩾Ala>Ser>	Val>Ileu, Gly, Thr;	Pro
,,	2	↑	,,	Val>Ileu>Gly, Thr;	Pro
,,	3	↓	,,	Val>Gly>Ileu>Thr;	Pro
,,	4	More sol.	,,	Gly>Val>Ileu⩾Thr;	Pro

* Observed similarly as in the foot-note of Table I.

** Prepared from the material obtained in 1951.

Some fractionations by ion-exchange resins were attempted, but they were not successful. However, several fundamental behaviors of arginine and clupeine towards the resins were observed, which would be useful in future and will be reported later.

EXPERIMENTAL

I-1. Preparation of Protamine Sulfates

A. Crude Nucleoprotamine from Milts: (*a*) Fresh milts of herring (*Clupea pallasii*) obtained at the coast of Mombetsu facing the Sea of Okhotsk, Hokkaido, Japan on May 7, 1947 were mainly used as the material. The fish was mature and said to be chiefly consisted of 7–8 years of age and also some of 5–6 years. About 10 hours after the landing of fish, milts were collected, washed with cold water, stored overnight at -5°, and then carried to a temporary laboratory packed with ice. The following separation of the sperm heads was started at about 58 hours after the landing.

According to a method which was almost similar to that of Felix (*27*), a suspension of ground milts in water was filtered through cloth, acidified with acetic acid, and the precipitate was centrifuged. Collected precipitate was washed with dilute acetic acid, water, acetone and ether, dried *in vacuo* first over sulfuric acid and then over phosphorus pentoxide. Slightly yellow powder (yield at the rate of *ca.* 600 g. from *ca.* 5 kg. of fresh milts) of crude sperm heads was thus obtained (mean values of analyses: N, 16.92 per cent by micro-Kjeldahl method; P, 2.3 per cent by micro-gravimetric method; residual "sulfated" ash, 1.1 per cent by Pregl's method).

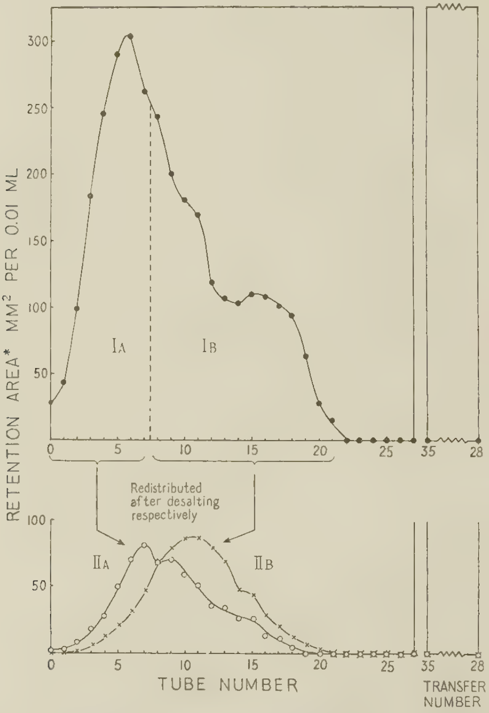

FIG. 2. Countercurrent distribution patterns of a clupeine sulfate.

Solvent system: Aqueous solution containing sodium *p*-toluenesulfonate (0.12 *M*) and NaCl (0.48 *M*) *vs.* *n*-butanol.

Temperature: 22–23°.

* The clupeine content in each tube is in proportion to the retention area (see experimental part, *III–3*) which was estimated as the mean value of 5 experiments.

Also material fish (*Clupea pallasii*) obtained in several places facing the Japan Sea in Hokkaido on March 31 (obtained at Yoichi, consisting of 5–6 years of age), April 3 (at Yoichi, 11–12 years), 12 (at Yoichi, age unknown) and 14 (at Reuke, mainly of 6–7 years), 1951, were used and similarly treated. The yield of sperm heads was always in *ca.* 11–15 per cent.

(*b*) Mature milts of salmon (*Oncorhynchus keta*) obtained in Chitose on a branch of the River Ishikari, Hokkaido on October 2, 1950 and sperm of rainbow-trout (*Salmo irideus*) obtained in the Okutama fish-farm near Tokyo, in every winter since 1951, were frozen and used for the preparation of nucleosalmine (yield *ca.* 16–18 per cent) and nucleoiridine (yield *ca.* 7 per cent) respectively.

B. Protamine Picrate from Crude Nucleoprotamine: Mainly according to the method of Rasmussen (*6*), dried powder of sperm heads obtained was repeatedly extracted by stirring with 0.2 N HCl (100 ml. per 10 g. of dried sperm heads) for 50 minutes. To each acid extract $M/8$ sodium picrate solution was added to precipitate protamine. Yellow precipitate from each extract was filtered after leaving it to stand several hours, washed with dilute sodium picrate solution, then with a small amount of ice water (or of $N/20$ sodium carbonate solution in some cases) and was dried *in vacuo* (total yield of picrate: *ca.* 25–50 per cent of the dried sperm heads). All the above treatments were carried out while cooling with ice, as long as protamine was in contact with acid.

C. Protamine Sulfates Both from the More and the Less Soluble Picrates: (a) Crude clupeine picrate (for an example, 24.5 g.) was dissolved into 67 per cent (*w/w*) aqueous acetone (*ca.* 230 ml. was used) and the solution was centrifuged to remove some yellow insoluble matter. The clear yellowish orange solution was left overnight or more in a cold room (0–1°), or in some cases put into a cold room after the addition at room temperature (17°) of a quantity of water (*ca.* 9 ml.) which was just enough to make it cloudy. The yellow supernatant solution (the more soluble part) was separated by decantation from the lower layer of brownish orange viscous oil (the less soluble part). On adding 2 N H_2SO_4 (30 ml.) and alcohol (200 ml.) while cooling to the supernatant, crude clupeine sulfate was precipitated which was centrifuged and washed with absolute alcohol. The precipitate was dissolved in water and purified by repeated precipitation with 2 N H_2SO_4 and alcohol, and for the last precipitation acetone was used instead of alcohol. The more soluble clupeine sulfate was thus obtained as colorless glittering powder (1.16 g.).

On the other hand, the residual viscous oil consisting of the less soluble picrate was dissolved into 67 per cent aqueous acetone (65 ml.) to produce a clear orange solution. From the solution the less soluble clupeine sulfate (5.22 g.) was obtained also as colorless glittering substance by a similar treatment of purification as described above. In this case, however, a considerable amount of substance was remained undissolved in water and it was discarded.

In order to effect the elimination of yellow color of crude clupeine sulfate due to picric acid contamination, such treatments as passing the aqueous solution through alumina or anion exchange resin (SO_4^{--}-form) column, or as thorough washing with

67 per cent aqueous acetone, were sometimes made with success.

(b) Almost in similar manner, the more soluble (0.31 g.) and the less soluble (3.25 g.) salmine sulfates were obtained respectively as colorless glittering powder from crude salmine picrate (for an example, 14.0 g.). In the case of iridine picrate (3.50 g.), its solution in 67 per cent aqueous acetone (150 ml.) produced no precipitate when left in a cold room. From the solution, colorless glittering substance of iridine sulfate (2.02 g.) was obtained unfractionated.

D. Analyses and Absorption Spectra of Protamine Sulfates:* Nitrogen content was determined by micro-Kjeldahl method under the condition of digestion recommended by Van Slyke *et al.* (*28*). It was 24.0±0.2 per cent for a sample of the less soluble clupeine sulfate dried *in vacuo* over $CaCl_2$ or concentrated H_2SO_4 (24.7±0.2 per cent for the moisture-free specimen). As for another sample of the less soluble clupeine sulfate, the nitrogen content was $23.7_5 \pm 0.1_5$ per cent for moisture-free specimen dried to constant weight *in vacuo* at 110° over P_2O_5 (moisture content 4.4_5 per cent). No appreciable amount of "sulfated" ash was found in this specimen.

The nitrogen content value was 23.3±0.2 per cent and 22.5±0.1 per cent respectively for sulfate specimens of the less soluble salmine and of iridine dried *in vacuo* over $CaCl_2$ or concentrated H_2SO_4.

Absorption spectra of some specimens of protamine sulfates were observed in their aqueous solutions with a Beckman quartz spectrophotometer, Model DU (*cf.* Fig. 1).

I–2. Preparation of Clupeine and Iridine Picrates without the Use of Acid

A. Sperm Heads from Suspension of Sperm or Milts in Dilute Salt Solution: (a) To a finely ground material of frozen milts of herring (for an example, 410 g. of milts obtained on April 14, 1951 and transported frozen to our laboratory in Tokyo) a solution (1.6 litre) of 0.05 *M* sodium citrate adjusted to pH 7 with HCl was added and stirred. After filtration of the mixture through cloth, the filtrate was centrifuged (4,000 r.p.m. for 10 minutes). The precipitate was again stirred with the citrate solution (1.6 litre) for 30 minutes and the suspension was centrifuged at a lower speed (1,300 r.p.m. for 1–2 minutes). Dark brown matter precipitated down (probably consisted of tissue debris) was discarded and the supernatant cloudy solution was further centrifuged at a higher speed (4,000 r.p.m. for 5 minutes). The material chiefly consisting of sperm heads was thus collected. It was white although the yeild (*ca.* 6 per cent usually when dried) was less compared with that by the method using acetic acid.

(b) Milts of rainbow-trout were similarly treated and sperm heads were collected.

B. Protamine Picrates from Sperm Heads through Treatment with Cupric Salt: (a) Wet sperm heads of herring mentioned in the above were added to 2 *M* NaCl solution (4 litre, containing 0.01 *M* sodium citrate) while vigorously stirring to yield highly viscous solution. Under continuous strong stirring, a saturated solution of cupric sulfate (400 ml.) was then added to the solution obtained above, to transform nucleoclupeine into soluble clupeine sulfate and insoluble copper nucleate. Such a strong stirring seemed to be necessary to obtain a good yield of clupeine sulfate. The capsule-like substance of copper nucleate, which is formed without strong stirring during the reaction, would probably enclose the nucleoclupeine in it and interfere with further extraction.

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* The analyses and the determination of absorption spectra were carried out by Drs. M. Kimura and Y. Kawanishi in our laboratory, to whom the authors are grateful.

After standing overnight, the precipitate of copper nucleate (*ca.* 23.2 g. when dried after washing with alcohol and ether) was filtered off and washed with water. *M*/8 Sodium picrate solution (400 ml.) was added to the combined filtrate and washings (6 *l*.), and yellow clupeine picrate (12.6 g.) was formed.

Cupric chloride was also used instead of the sulfate with similar results in yield.

(b) By a similar treatment of frozen sperm of rainbow-trout (for an example, formed from 250 ml. of fresh sperm obtained on March 7, 1952) picrate of iridine (5.1 g.) was obtained. The poorer yield might be due to insufficient stirring which is important at the time of addition of cupric salt, as was emphasized in the above.

All the treatments involved in this section (*I–2*) were made while cooling with ice or in a cold room. The protamine picrates thus prepared were transformed into their sulfates as described before.

II. Amino Acid Composition of Protamines

A. Specimens of Hydrolysate: Protamine sulfate (for an example, 150 mg.) was hydrolyzed by heating at 110–120° in sealed tube for 10–30 hours with 20 per cent HCl (3 ml. of analytical grade or redistilled HCl). The hydrolysate was repeatedly treated by evaporation to dryness followed by addition of distilled water, to remove HCl as possible. From the mixture most of arginine, always present in a large amount, was removed in advance by one of the following methods to obtain a reliable result of analysis of other component amino acids.

(a) To the mixture flavianic acid (170 mg. as crystalline trihydrate) was added (total volume of the resulted solution being 10 ml.) and the precipitate of arginine monoflavianate was removed. Before or after the treatment with flavianic acid, $BaCO_3$ (50 mg.) was added to the solution to remove most of sulfate ion, and the concentrated solution was then paper-chromatographed without any interfering effect of such ion.

(b) In a case of iridine sulfate, arginine was removed by passing the solution through a column of strong basic ion-exchange resin, Amberlite IRA-400 (OH-form). Only arginine passed through the column while monoamino acids were retained in it and they were then eluted with 1 *N* HCl. Most part of HCl was removed from the acid eluant by repeated evaporation and the residual concentrate was used for analysis*.

Each specimen of hydrolysate prepared either by method (a) or (b) still contained always a small quantity of arginine.

B. Analyses of Component Amino Acids by Paper Chromatography: Detection of most of amino acids contained and comparison of their content from the visual total color

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* The composition analysis of this specimen was carried out by Dr. K. Miura in this institute, to whom the authors are grateful.

(the area and intensity) of spots were made on two-dimensional paper chromatogram revealed with ninhydrin. The total color of spot of the same quantity of each monoamino acid was assumed to be the same. Phenol-0.1 per cent NH_3 (6:1, *w/w*, 0.04 per cent *8*-hydroxyquinoline was added) was employed as the first solvent and collidine-water (3:2 or 5:3, *v/v*) or *n*-butanol-acetica cid-water (4:1:1, *v/v*) as the second solvent. The Toyo filter paper No. 50 (40×40 cm.) was used and revealed as usual by spraying 0.2 per cent ninhydrin in aqueous butanol followed by heating at 100° for 5 minutes. The results were shown in Table I.

The following supplementary tests were also made.

(*a*) *Identification of Isoleucine* It was found possible to distinguish between leucine and isoleucine by one-dimensional chromatography on the Toyo filter paper No. 50 (2×40 cm.), using the toluene- system solvent (mixed solution of toluene, pyridine, ethylene chlorohydrin and 0.8 *N* NH_4OH in the volume ratio 5:1:3:0.3) which is commonly used for separation of DNP-amino acids (*29*). On the chromatogram the R_f values of isoleucine and leucine showed 0.18_5 and 0.22 respectively. The spot of "leucine" part of clupeine hydrolysate was concluded to be isoleucine from its R_f value (0.19). Also a chromatography with development of longer distance on the Toyo filter paper No. 52 (2×60 cm.) using *n*-butanol- acetic acid- water (4:1:5, *v/v*) as a solvent could distinguish between the two amino acids, leucine moving faster than isoleucine. By this test it was also concluded that isoleucine and not leucine was contained in the hydrolysate of iridine.

(*b*) *Non-existence of Histidine, Tyrosine, Cystine and Methionine in the Specimens of Protamines* Paper strips of one-dimensional chromatography of each protamine hydrolysate were tested respectively for histidine and tyrosine by applying Pauly's reaction (*30*), for cystine and methionine by platinic iodide reaction (*30*) and Feigl's sodium azide-iodine reaction (*30*), and for cystine also by sodium nitroprusside reaction (*30*). All the results did not show any positive existence of the amino acids tested.

III–1. Tentative Fractionation of Clupeine into Four Parts by Making Use of the Solubility Difference among Their Picrates

The clupeine picrate used for the present fractionation was prepared from the second extract with 0.2 *N* HCl of a dried precipitate of sperm heads collected by acetic acid treatment of milts obtained on April 12, 1951. The picrate (6.88 g.) was added to a quantity (*ca.* 70 ml.) of 67 per cent aqueous acetone just enough to dissolve the picrate at room temperature and the solution was left for *ca.* 40 hours at 0°. An oily fraction (L) separated down from the supernatant layer (M). On repeating the similar treatment upon the separated fraction L, it was further fractionated into precipitate (fraction 1) and the supernatant (fraction 2). On the other hand, water was added to the layer M in such a quantity (*ca.* 5 ml.) just enough to initiate precipitation. After cooling with ice the precipitate (fraction 3) was separated from the supernatant (fraction 4). Four fractions of clupeine picrate thus obtained were transformed respectively into their sulfates and purified as described before (*I–1c*), and colorless glittering preparations were obtained respectively. The yield of sulfates was as follows:

	L		M	
	fraction 1	fraction 2	fraction 3	fraction 4
	less	soluble ←——→	more soluble	
Crude prep. (g.)	0.873	0.706	0.428	0.041
Purified prep. (g.)	0.593	0.458	0.271	0.012

Hydrolysate of each fraction of purified sulfate was chromatographed on paper similarly as described in the section II. The results are shown in Table II.

III–2. Countercurrent Distribution of Clupeine (*A Preliminary Experiment*)

A. Sample: The clupeine sulfate used for the present purpose was that prepared through picrate from the first extract with 0.2 N HCl of the same sperm heads as used in the preceding experiment (*III–1*). In this case, however, all the picrate was immediately transformed into a sulfate without fractionation.

B. Operation of Countercurrent Distribution: Countercurrent distribution was operated in an apparatus fitted with 28 glass tubes of Craig type (*31*), the volume of the upper phase and the lower being 10 ml. respectively. The solvent system used was that of two phases resulted from mixing 1 volume of *n*-butanol and 1 volume of aqueous solution of both 0.12 M sodium *p*-toluenesulfonate (recrystallized) and 0.48 M NaCl. The partition coefficient of clupeine sulfate in the solvent system was *ca.* 0.5 and remained almost constant when the concentration of the protamine sulfate was less than 0.2 per cent in the total volume of both phases. The coefficient showed, however, a tendency to decrease somewhat with an elevation of temperature, and hence the temperature must be kept constant throughout an operation. The temperature of the present experiment was kept at 22–23°.

Under these experimental conditions the clupeine sulfate (110 mg.) was distributed by a procedure of 35 transfers. The fractions obtained by a single withdrawal procedure (No. 28–35) were discarded as they contained no clupeine. To each of other tubes (No. 0–27) methanol (2.5 ml.), water (2.5 ml.) and ether (10 ml.) were added, and they were shaken to transfer all the clupeine into the aqueous phase. Amount of clupeine in each fraction was successfully determined by making use of a retention analysis (see later, *III–3*). The result thus obtained is shown in a curve (I_A–I_B) of Fig. 2.

On the basis of the distribution curve, two fractions were formed by combining the content of tubes No. 0–7 (corresponding to curve I_A) and No. 8–21 (I_B) respectively. Both fractions were desalted as described in the next paragraph (*C*) and clupeine was recovered in yields of 34.9 mg. and 33.7 mg. as sulfate respectively. Each part of the protamine was then redistributed under the same conditions. The resulting curves are also shown in II_A and II_B of Fig. 2.

C. Desalting by Ion-exchange Resins of Clupeine Solution Resulted from Countercurrent Distribution: Elimination of sodium *p*-toluenesulfonate and NaCl from the aqueous phase and regeneration of clupeine sulfate were performed satisfactorily in the following manner. When the combined aqueous solution was passed through a column of

Amberlite IRC-50 (XE-64) in the H-form (4 ml.), whole clupeine and a part of sodium ion were retained. After eluting sodium ion by washing the column with 1 *N* acetic acid, the clupeine was then eluted by 0.1 *N* HCl (30 ml.) while cooling with ice water. The acid effluent containing clupeine was neutralized with Amberlite IR-4B (OH-form) and lyophilized to yield clupeine hydrochloride, which was transformed as usual into its sulfate.

III–3. Retention Analysis of Clupeine

Minute quantities of clupeine in solution were estimated satisfactorily by modifying Wieland's retention analysis (*32*). A mixed developer of the following composition was used in the present analysis. Just before the use, 5 volumes of solution A (consisting of 30 ml. of methanol, 150 g. of glycerol and 250 ml. of water) and 1 volume of solution B (60 mg. of Ponceau-6 RF, 45 ml. of acetic acid and 35 ml. of water) were mixed.

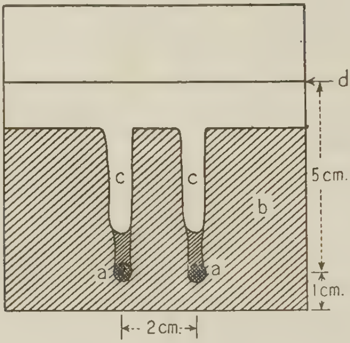

Fig. 3. Retention figures of clupeine.
a: Original spot b: Colored area c: Non-colored area (retention area) d: Solvent front

Clupeine solutions of various concentrations (containing quantities less than 5 μg. as sulfate in 0.005–0.02 ml. of water) were spotted, at a distance of 2 cm. between spots, 1 cm. above the lower side of a paper (the Toyo filter paper No. 50, 7 cm. long and suitably wide). In such a case of solution containing some salt as resulted from a countercurrent distribution, the spotted paper was once developed by distilled water and air-dried, thereby the salt moved with water while the clupeine remained on the original point. The paper was then developed by the freshly mixed Ponceau solution described in the above. Thus the retention figures corresponding to amounts of clupeine were obtained without any treatment of coloration (Fig. 3). The retention area was *ca.* 100 sq.mm. per 5 μg. of clupeine sulfate, and the relation of the quantity to the retained area was linear when it was less than 5 μg. The error of measurement defined as the coefficient of variation (standard deviation/arithmetical mean) was found to be *ca.* 0.04.

SUMMARY

1. Sulfates of protamines (clupeine, salmine and iridine) were prepared by various methods from fresh and mature milts or sperm of fishes (*Clupea pallasii*, *Oncorhynchus keta* and *Salmo irideus* correspondingly) obtained in Japan, with prudence during the course of extraction and purification.

2. The amino acid compositions of the protamine sulfates were studied qualitatively (*cf.* Table I). Glycine was discovered in clupeine.

3. Inhomogeneity was suggested for clupeine at least from two series of experiments, *i.e.* from comparison of the relative amino acid contents among fractionated preparations (*cf.* Table I and II) and from the counter-current distribution (*cf.* Fig. 2). Retention analysis with an acid dye, Ponceau-6RF, was also described as a simple determination method of protamine.

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REFERENCES

(*1*) Kossel, A., Protamine und Histone, Franz Deuticke, Leipzig und Wien (1929)

(*2*) Ando, T., and Iwai, K., Nucleic Acids and Nucleoproteins, Edited by Egami, F., Kyoritsu Shuppan Co., Tokyo, Vol. **1**, p. 378 (1951)

(*3*) Ando, T., Iwai, K., and Yamasaki, M., Chemistry of Proteins, Edited by Akabori, S., and Mizushima, S., Kyoritsu Shuppan Co., Tokyo, Vol. **3**, p. 89 (1955)

(*4*) Ando, T., Ishii, S., Hashimoto, C., Yamasaki, M., and Iwai, K., *Bull. Chem. Soc. Japan*, **25**, 132 (1952)

(*5*) Ando, T., Iwai, K., Yamasaki, M., Hashimoto, C., Kimura, M., Ishii, S., and Tamura, T., *Bull. Chem. Soc. Japan*, **26**, 406 (1953)

(*6*) Rasmussen, K. E., *Z. physiol. Chem.*, **224**, 97 (1934)

(*7*) Pollister, A. W., and Mirsky, A. E., *J. Gen. Physiol.*, **30**, 101 (1946)
(*8*) Waldschmidt-Leitz, E., and Günther, E., *Makromol. Chem.*, **2**, 120 (1948)
(*9*) Waldschmidt-Leitz, E., Kühn, K., and Zinnert, F., *Experientia*, **7**, 183 (1951)
(*10*) Block, R. J., Bolling, D., Gershon, H., and Sober, H. A., *Proc. Soc. Exptl. Biol. Med.*, **70**, 494 (1949)
(*11*) Felix, K., Fischer, H., Krekels, A., and Rauen, H. M., *Z. physiol. Chem.*, **286**, 67 (1950)
(*12*) Kuroda, Y., *J. Biochem.* (*Japan*), **38**, 115 (1951)
(*13*) Stedman, E., and Stedman, E., *Philos. Trans. Royal Soc. London*, **B235**, 565 (1951)
(*14*) Šorm, F., and Šormová, Z., *Collection Czechoslov. Chem. Communs.*, **16**, 207 (1951)
(*15*) Block, R. J., and Bolling, D., *Arch. Biochem.*, **6**, 419 (1945)
(*16*) Tristram, G. R., *Nature*, **160**, 637 (1947); Advances in Protein Chem., **5**, 129 (1949)
(*17*) Hamer, D., and Woodhouse, D. L., *Nature*, **163**, 689 (1949)
(*18*) Velick, S. F., and Udenfriend, S., *J. Biol. Chem.*, **191**, 233 (1951)
(*19*) Blackburn, S., and Robson, A., *Biochem. J.*, **54**, 295 (1953); Corfield, M. C., and Robson, A., *ibid.*, **55**, 517 (1953)
(*20*) Felix, K., Fischer, H., and Krekels, A., *Z. physiol. Chem.*, **289**, 127 (1952)
(*21*) Felix, K., Fischer, H., Krekels, A., and Mohr, R., *Z. physiol. Chem.*, **287**, 224 (1951)
(*22*) Iso, K., Kitamura, T., and Watanabe, I., *J. Chem. Soc. Japan* (*Pure Chem. Sec.*), **75**, 342 (1954)
(*23*) Ui, N. and Watanabe, I., *J. Chem. Soc. Japan* (*Pure Chem. Sec.*), **74**, 647 (1953)
(*24*) Ui, N., *Reports of the Institute of Science and Technology, Univ. of Tokyo*, **8**, 255 (1954)
(*25*) Waldschmidt-Leitz, E., and Voh, R., *Z. physiol. Chem.*, **298**, 257 (1954)
(*26*) Rauen, H. M., Stamm, W., and Felix, K., *Z. physiol. Chem.*, **292**, 101 (1953); Felix, K., Lecture in Tokyo, Japan, Nov. 1955
(*27*) Felix, K., and Mager, A., *Z. physiol. Chem.*, **249**, 111 (1937)
(*28*) Hiller, A., Plazin, J., and Van Slyke, D. D., *J. Biol. Chem.*, **176**, 1401 (1948)
(*29*) Biserte, G., and Osteux, R., *Bull. soc. chim. biol.*, **33**, 50 (1951)
(*30*) Block, R. J., Paper Chromatography, Academic Press Inc., New York, pp. 63, 64 (1952)
(*31*) Craig, L. C., and Post, O., *Anal. Chem.*, **21**, 500 (1949)
(*32*) Wieland, T., and Fischer, E., *Naturwiss.*, **35**, 29 (1948); Wieland, T., and Wirth, L., *Angew. Chem.*, **63**, 171 (1951)
(*33*) Markham, R., and Smith, J. D., *Nature*, **164**, 1052 (1949)

The Journal of Biochemistry, Vol. 44, No. 5, 1957

ON THE INTERACTION OF CYCLIC AMINO ACIDS WITH ASCORBIC ACID

III. AN EFFECTIVE METHOD FOR THE PREPARATION OF KYNURENINE

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(Received for publication, December 13, 1956)

It has been reported that tryptophan can be converted to kynurenine merely by bubbling a stream of air through tryptophan solution in the presence of ascorbic acid (*1*, *2*), the yield being about 18 per cent. The yield of Otani's analogous method using potassium permanganate as oxidizing agent, is 4.6 per cent (*3*), and that of Amano's method, using ozone, is 10 per cent (*4*).

As our method seems more effective than the others, we have attempted to prepare crystalline kynurenine by this method.

The methods of analysis of kynurenine and tryptophan have been described earlier.

EXPERIMENTAL AND RESULTS

Experiment I—0.2 g. of L-tryptophan, 0.3 g. of L-ascorbic acid, 0.04 ml. of 10 per cent $CuSO_4$ and 25 ml. of aq. dest. were placed in a Foline-tube, incubated in a water bath at 37°, and air was bubbled through the mixture for 6 hours. After the reaction had ended, we separated amorphous kynurenine by Kotake-Kawase's method (*5*). The substance was further purified with countercurrent apparatus with 60 tubes, butanol saturated with water being used as solvent. But, as kynurenine did not distribute in this solvent, the author evaporated the lower layers of the first to fourth tubes to dryness, extracted the residue with alcohol, and obtained yellow needle-like crystal. The yield was 36 mg., corresponding to 18 per cent of tryptophan used.

Experiment II—In this experiment, the solvent system was replaced by butanol-water-acetic acid (5:4:1), the treatment by Kotake-Kawase's method was omitted and kynurenine was separated directly from the reaction mixture by means of countercurrent distribution.

Figs. 1 and 2 show the distribution curves obtained for kynurenine and tryptophan. The partition coefficients of tryptophan and kynurenine could be calculated to be

$$K_{try}=2.14 \text{ and } K_{kyn}=1.67$$

by the equation,

$$N=n\frac{K}{K+1}, \quad K=\frac{N}{n-N}$$

where, K is the partition coefficient, N, the tube number which contains the highest concentration of solute, and n, the total number of tubes used in the experiment.

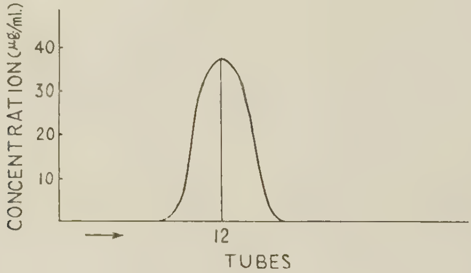

Fig. 1. Distribution curve of kynurenine.

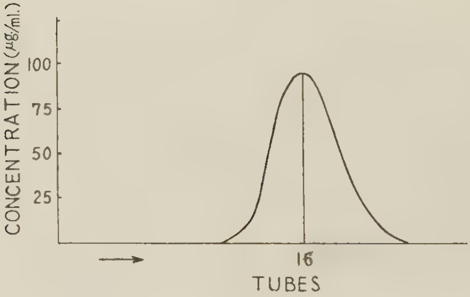

Fig. 2. Distribution curve of tryptophan.

Though tryptophan and kynurenine have similar R_f value, and we can scarcely identify them with paper partition chromatography, there is a definite difference of partition coefficient between them. But, to identify them perfectly by countercurrent method, a hundred or more tubes must be employed, and it seems more advisable to select other solvent system.

Experiment III—In the oxidation of D-tryptophan, no difference was observed from that of its L-isomer (Fig. 3). Oxdiation product, which has been obtained in the same way, has R_f value of 0.47 (solvent: butanol-water-acetic acid=5:4:1), which is equal to the R_f value of kynurenine in the same solvent. It shows positive ninhydrine reaction, and its absorption curve is very similar to those of kynurenine (Fig. 4). Its m.p. is 197°.

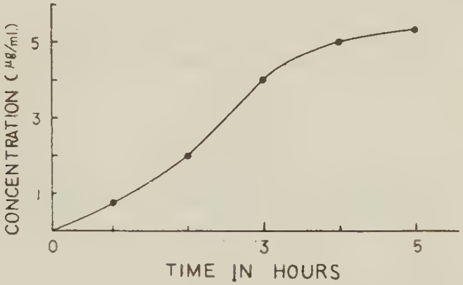

FIG. 3. Curve of kynureine converted from D-tryptophan when air-bubbling was carried out with ascorbic acid.

The solution: D-tryptophan, 10 mg.; ascorbic acid, 20 mg.; $CuSO_4$, 0.04 ml.; aq. dest., 50 ml.

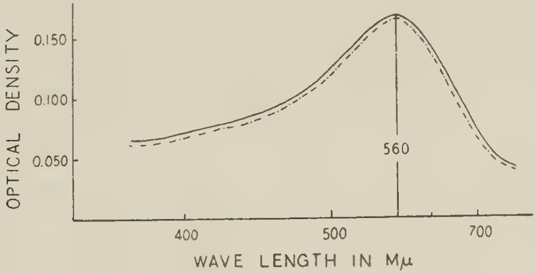

FIG. 4. Absorption curves.

Full line: Kynurenine

Brocken line: the oxidation product

The absorption curve of the oxidation product corresponds roughly to that of kynurenine.

A comparative study on formylkynurenine, and further survey on the decomposition process are under consideration.

SUMMARY

1. Kynurenine has been prepared by the interaction of tryptophan and ascorbic acid in the presence of air, and purified by Kotake-Kawase's method and countercurrent separation.

2. D-tryptophan behaves in the same way as its L-isomer.

REFERENCES

(*1*) Torii, K., and Moriyama, T., *J. Biochem.*, **42**, 193 (1955)

(*2*) Torii, K., and Moriyama, T., *J. Biochem.*, **42**, 201 (1955)

(*3*) Otani, Z., Kagakuzikkengaku (in Japanese), Kawade, Tokyo, Vol. **13**, 305 (1945)

(*4*) Amano, T., *Med. J. Osaka Univ.*, **2**, 45 (1950)

(*5*) Kawase, M., *Osaka Igaku Zassi*, **31**, 1404 (1942)

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ON THE INTERACTION OF CYCLIC AMINO ACIDS WITH ASCORBIC ACID

IV. EXPERIMENTS IN VIVO

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(Received for publication, December 13, 1956)

In the course of our studies on the metabolism of tryptophan, it has been found that tryptophan reacts with ascorbic acid.

Similar results were also obtained with tyrosine and histidine. Further, we have obtained crystals of kynurenine by bubbling a stream of air through the mixed solution of tryptophan and ascorbic acid, and have studied the dependence of its yield upon the concentrations of ferric and cupric ions and of hydrogen peroxide added to the solution. These results have been published at the last general session of the Biochemical Society of Japan (*1*, *2*, *3*).

The present experiments were undertaken to decide whether such a process does also occur *in vivo*.

About the fate of tryptophan in the case of ascorbic acid deficiency, ample works have been contributed by the biochemists of Osaka University. Their results show that the deficiency inhibits the formation of kynurenine, and indole lactic acid and tryptophan are excreted unchanged (*4*).

In the present experiments, the author examined these substances in urine under the condition of ascorbic acid saturation.

The analysis was done by modified Roe's method (using 2,4-dinitrophenylhydrazine) for ascorbic acid (*5*), by Spies' method (using *p*-dimethylaminobenzaldehyde) for tryptophan (*6*), and by Itou-Mochizuki's method for kynurenine (*7*), respectively.

Polished rice meal was prepared by kneading polished rice powder with 10 per cent of its weight of filter paper in hot water, and normal diet was composed of a definite amount of bean-curd refuse and vegetables.

100 mg. of ascorbic acid was injected daily, until the excreted amount of ascorbic acid in urine began to be constant. Then ascorbic acid saturation was regarded as established and the administration of

tryptophan was commenced.

EXPERIMENTAL AND RESULTS

Experiment I.—25 mg. of tryptophan was administered to normal diet rabbit and polished rice diet rabbit by subcutaneous injection.

After 6 hours samples of urine were removed from time to time and analysed for tryptophan and kynurenine (Table I).

Table II shows the results obtained, when, in addition to 25 mg. of tryptophan, 100 mg. of ascorbic acid was injected intraveneously through *vena auricularis* at the same time. These data suggest that larger amount of kynurenine is excreted by polished rice diet rabbit.

Table I

Change of the Excreted Amount of Tryptophan and Kynurenine in Urine with Time After the Administration of 25 mg. of Tryptophan to Normal Diet Rabbit

	0	1	2	3	4	5	6 *hrs.*
Tryptophan	152	516	572	156	80	74	74
Kynurenine	73	332	236	80	56	48	75

μg/ml.

Change of the Excreted Amount of Tryptophan and Kynurenine in Urine with Time After the Administration of 25 mg. of Tryptophan to Polished Rice Rabbit

	0	1	2	3	4	5	6 *hrs.*
Tryptophan	89	59	29	18	17	24	32
Kynurenine	16	18	98	106	78	83	77

μg/ml.

Experiment II.—Table III shows the amounts of tryptophan, kynurenine, and ascorbic acid in total urine excreted in a day, respectively before and after the subcutaneous injection of 25 mg. of tryptophan to polished rice diet rabbit. As a control, the case of normal diet rabbit is given in Table IV (*8*).

These results show that the amount of kynurenine as well as that of tryptophan is larger in the urine of polished rice diet rabbit than that of normal diet rabbit.

The amounts of ascorbic acid are reduced in both cases by 37 mg. to 50 mg. when tryptophan is administered.

DISCUSSION

Table V shows the rates of the amounts of tryptophan and kynurenine recovered from urine to the amount of tryptophan administered in each experiment. As shown above, kynurenine formation increases by the administration of ascorbic acid. This result is contradictory to the experimental results obtained at the biochemical institute of Osaka University under the condition of ascorbic acid deficiency.

TABLE II

Change of the Excreted Amount of Tryptophan and Kynurenine with Time in Urine after the Administration of 25 mg. of Tryptophan and 100 mg. of Ascorbic Acid to Polished Rice Diet Rabbit

	1	2	3	4	5	6 *hrs.*
Tryptophan	180	83	45	56	54	54
Kynurenine	240	600	705	690	660	540

μg/ml.

TABLE III

Change of the Excretion of Tryptophan, Kynurenine and Ascorbic Acid by the Injection of 25 mg. of Tryptophan to Polished Rice Diet Rabbit Saturated with Ascorbic Acid

Days	Total urine *ml./day*	Tryptophan *mg.*	Kynurenine *mg.*	Ascorbic acid *mg.*
15	68	4.2	2.3	54.0
16	127	4.3	2.9	50.4
Injection: 25 mg. of tryptophan				
17	105	7.1	7.2	13.1
18	91	5.2	4.4	41.5
19	110	4.2	2.4	50.5

TABLE IV

Change of the Excretion of Tryptophan, Kynurenine and Ascorbic Acid by the Injection of 25 mg. of Tryptophan to Normal Diet Rabbit Saturated with Ascorbic Acid

Days	Total urine *ml./day*	Tryptophan *mg.*	Kynurenine *mg.*	Ascorbic acid *mg.*
15	130	8.1	3.5	72.4
16	110	7.9	3.2	75.8
Injection: 25 mg. of tryptophan				
17	100	5.1	5.6	22.1
18	100	8.0	3.7	70.5
19	120	8.5	3.5	70.3

TABLE V

Percentage of the Amounts of Tryptophan and Kynurenine Recovered from Urine to the Amount of Tryptophan Administered in Each Experiment

Condition of rabbit	Tryptophan %	Kynurenine %	Total %
Normal diet	16.7	9.3	26.0
Polished rice diet	2.2	6.3	8.4
Polished rice diet, injected ascorbic acid*	6.8	62.4	69.4
Normal diet, saturated with ascorbic acid	20.0	22.4	42.4
Polished diet, saturated with ascorbic acid	28.5	28.8	57.3

* Once injected 100 mg. of ascorbic acid.

If ascorbic acid functioned as an activator of enzyme system, larger amount of kynurenine would have to be excreted in the case of ascorbic acid saturation rather than in that of single administration of ascorbic acid. The fact that no difference is observed between these two cases, may suggest that ascorbic acid does not activate the enzyme system, but it reacts directly with the tryptophan molecule to split the pyrrole ring. If we express, in moles, the decrease of ascorbic acid in urine by the administration of tryptophan, the molar ratio of tryptophan administered to the decrease in urine of ascorbic acid is 1 to 1 in the case of polished rice diet rabbit and about 1 to 2 in the case of normal diet rabbit.

At present, this difference remains unexplained, but it seems probable that in the latter case a part of ascorbic acid would be utilized in another metabolitic process, such as opening another nucleus, *e. g.*, benzene ring of kynurenine.

SUMMARY

1. As *in vitro*, ascorbic acid injection increases the formation of kynurenine *in vivo*.

2. Administration of tryptophan reduces the excretion of ascorbic acid in urine, the molar ratio of tryptophan administered to the decrease of ascorbic acid in urine being 1 : 1–2.

These results are in accord with the *in vitro* experiments described in the earlier reports.

REFERENCE

(*1*) Torii, K., and Moriyama, T., *J. Biochem.*, **42**, 193 (1955)
(*2*) Torii, K., and Moriyama, T., *J. Biochem.*, **42**, 201 (1955)
(*3*) Moriyama, T., *J. Biochem.*, **44**, 289 (1957)
(*4*) Sakagoshi R., *Vitamins*, **7**, 152 (1954)
(*5*) Roe, J. H. and Kuether, C. A., *J. Biol. Chem.*, **147**, 399 (1943)
(*6*) Spies, J., and Chambers, D. C., *Anal. Chem.*, **21**, 1249 (1949)
(*7*) Mochizuki, K., *J. Nara Med. Assoc.*, **4**, 17 (1953)
(*8*) Mochizuki, K., and Moriyama, T., *J. Nara Med. Assoc.*, **6**, 73 (1955)

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BIOSYNTHESIS OF THREONINE FROM HOMOSERINE

VI. HOMOSERINE KINASE*

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Previous papers of this series (*1*, *2*) presented evidence that the enzymic conversion of L-homoserine to L-threonine involves two distinct steps. These are catalyzed by separate enzymes from bakers' yeast. The first step is the phosphorylation of homoserine in the presence of ATP**, resulting in the formation of *O*-phosphohomoserine. A new enzyme which catalyzes this phosphate transfer was designated as homoserine kinase (ATP-homoserine transphosphatase). The importance of this reaction in threonine biosynthesis was established from studies of the over-all reaction (*2*).

This paper presents further studies on the properties of the enzyme and its partial purification.

MATERIALS AND METHODS

Materials—Bakers' yeast was obtained commercially from Sankyo Co., Ltd. DL-Homoserine and L-homoserine were prepared as described previously (*3*). The ATP used was either the barium salt separated from canine muscle (*4*) or the sodium salt from Schwarz Laboratories Inc. Both salts were approximately 80 per cent pure with respect to acid-labile phosphate content. The barium salt of ATP was converted to the sodium salt by ion exchange treatment, followed by neutralization.

Assay of Enzyme—As noted previously (*2*), when the mixture containing homoserine, ATP and homoserine kinase was incubated stoichiometric disappearance of acid-labile phosphate and formation of *O*-phosphohomoserine were observed. The enzyme activity, therefore, could be determined by following the disappearance of acid-labile phosphate. In the experiments described here, a more convenient and rapid manometric assay

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** The following abbreviations are used: ATP, adenosinetriphosphate; PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetic acid.

in a Warburg apparatus using a CO_2-bicarbonate system was used. The procedure was essentially the same as reported by Cohen (*5*) in the case of gluconokinase, except that, in most experiments with partially purified enzyme, the reaction was carried out in an atmosphere of air-CO_2 instead of nitrogen-CO_2. The oxygen consumption of the enzyme preparation was negligible even in the presence of homoserine.

The reaction mixture contained, unless otherwise noted, 25 μM of DL-homoserine, 12 μM of ATP, 10 μM of $MgSO_4$, 20 to 40 μM of NaF, 50 μM of $NaHCO_3$ and kinase preparation containing 0.3 to 0.5 mg. of protein-nitrogen in a total volume of 2.0 ml. Usually 0.5 ml. of ATP solution was pipetted into the side arm, and, after equilibration in air-CO_2, the reaction was started by tipping the contents of the side arm. The readings without added homoserine were taken as control.

As shown in Table I, the CO_2 evolution approximately paralleled the disappearance of acid-labile phosphate which represents the formation of *O*-phosphohomoserine.

TABLE I

Comparison of CO_2 Evolution and Disappearance of Acid-labile Phosphate

Time	Manometric detn.	$-\Delta$ Acid-labile P
min.	μM	μM
10	0.87	1.00
20	1.69	1.83
30	2.49	2.81
50	3.95	4.58

The reaction system was that noted in the text. After reading a manomater, the reaction was stopped by adding 2 ml. of 10 per cent trichloroacetic acid at each reaction time, and the disappearance of acid-labile phosphate was determined by the procedure described previously (*2*).

RESULTS AND DISCUSSION

Purification of Enzyme—Fraction I was separated from acetone-dried bakers' yeast by the procedure described previously (*1*). This was further purified, after being lyophilized in some experiments. All procedures were carried out at about 3°. One gram of lyophilized powder was dissolved in 25 ml. of water at pH 7.0. The turbid solution was clarified by centrifugation for 15 minutes at 10,000 × *g*. In the case of nonlyophilized enzyme, this procedure was omitted. To the yellow supernatant fluid was added approximately 3 ml. of alumina $C\gamma$ gel which had been aged about two years (dry weight 35 mg. per ml.) After 10 minutes of stirring at pH 7.0, the gel was removed by centrifugation for 10 minutes at 8,000 × *g* and discarded. The clear supernatant solution was brought to 0.20 saturation with powdered ammonium sulfate (1 saturation; 72 g. added to 100 ml.). This was stirred for 20 minutes and then centrifuged for 15 minutes at 10,000 × *g*. To the supernatant ammonium sulfate was added to 0.35 saturation and stirred for 20 minutes. The precipitate was collected by centrifugation and washed with 0.4 saturated water. This was then redissolved in water and dialyzed for 24 hours against two changes of distilled water (1,000 ml. each).

At this stage, the enzyme activity decreased about 30 per cent during a week when stored at −5°. Considerable loss in activity was also caused by lyophilizing the solution.

A typical result of the purification is summarized in Table II. This experiment was carried out without lyophilization at any stage, and the yield of kinase was the best of several experimental runs. The treatment with alumina did not increase the specific activity greatly, but removed interfering ATPase. The remaining ATPase activity was almost completely inhibited by the use of fluoride (0.02 *M*).

TABLE II

Summary of Purification of Kinase from Yeast

Treatment	Total units†	Specific activity‡
Acetone powder ext.	(310)*	(0.3)*
pH 4.8 treated	(290)*	(0.4)*
$(NH_4)_2SO_4$ 0.4 ppt. *(Fraction I)*	230	1.33
Alumina treated	207	2.51
$(NH_4)_2SO_4$ 0.2 to 0.35 ppt.	180	4.79

† Total units: CO_2 evolved in microlitre per minute under the conditions described in the text.

‡ Specific activity: CO_2 evolved in microlitre per minute per mg. of protein-nitrogen.

* These values are not reliable because of low activities of the extracts and of contamination with interfering enzymes.

Enzyme Properties—The reaction was found to follow zero order kinetics for at least the first 20 minutes and was proportional to enzyme concentration up to 1.0 mg. protein-nitrogen per vessel (Fig. 1). The apparent pH optimum ranges from 7.0 to 7.5, although this could not represent the true reaction rate at each pH level, since quantitative CO_2

evolution is not expected below pH 7.5 in this system (*6*) (Fig. 2). For convenience, assays were usually carried out at pH 7.5.

Substrate Requirements—The Michaelis constant for ATP, derived from Lineweaver-Burk plots, was found to be of the order of $10^{-2}M$ (Fig. 3). The effect of varying homoserine concentration is illustrated in Fig. 4. Higher concentrations of the substrate appeared to be rather inhibitory to the enzyme action. At present there is no evidence to determine whether this is due to the inhibitory effect of the substrate or the reaction product. It must be noted that these experiments were carried out at ATP concentrations up to 12 mM, and this phenomenon seemed to be independent of ATP concentration under the test conditions.

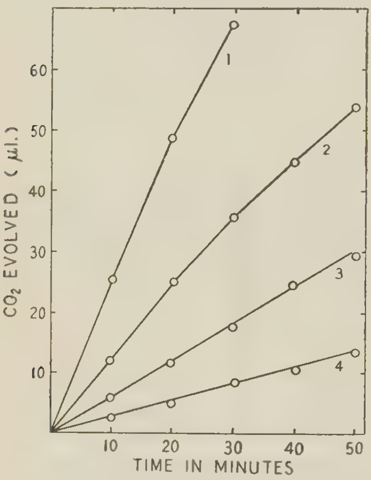

Fig. 1. Effects of time and enzyme concentration. The assay system was the same as described in the text, except that the ATP concentration was 6 μM per vessel. Curve 1 was 1.0 mg. protein-N per vessel, in curves 2,3 and 4 the enzyme protein was 0.5, 0.25 and 0.125 mg., respectively.

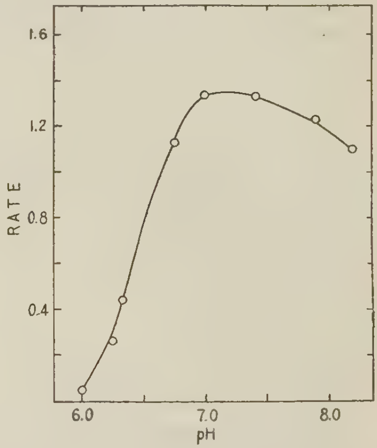

Fig. 2. Apparent optimum pH. The pH was adjusted by varying the concentration of sodium bicarbonate and CO_2 pressure according to Umbreit (*8*). Each vessel contained 25 μM of DL-homoserine, 12 μM of ATP, 10 μM of $MgSO_4$, 40 μM of NaF, $NaHCO_3$ and kinase preparation in a total volume of 2.0 ml.

Metal Activation—It was found that the kinase was stimulated by magnesium and some other divalent metals, the activation being of the order of 200 per cent at the most. This was found to be rather erratic depending upon the enzyme preparation. Typical results are summarized in Table III. Prolonged dialysis of the enzyme against distilled water did not increase the metal activation. However, there is an indication of a definite requirement for metal ions. As can be seen in Table IV, the kinase was markedly inhibited by 0.005 *M* EDTA, and this was found to be reversed by the addition of metals. The effects of Mn^{++} and Zn^{++} appeared to be highly variable depending on the experimental conditions. If metals were preincubated with the enzyme solution for 15 minutes at 37°, the reaction was stimulated by Mg^{++} and Mn^{++}, but inhibited by Zn^{++} (Table III, Experiment 2). If the

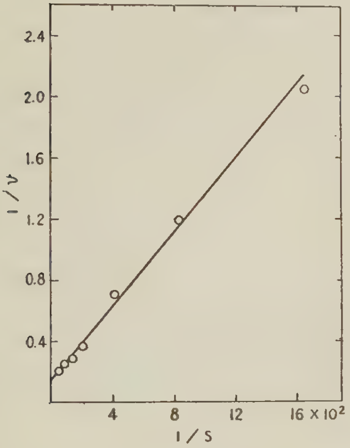

Fig. 3. Effect of ATP concentration. Ordinate, reciprocal of velocity, where v = CO_2 evolved (μl.) per minute; abscissa, reciprocal of ATP concentration. Reaction system was the same as noted in Fig. 2 and in the text, except that ATP solution was pipetted into the main compartments of the Warburg vessel, and the reaction was started by tipping enzyme solution from the side arm.

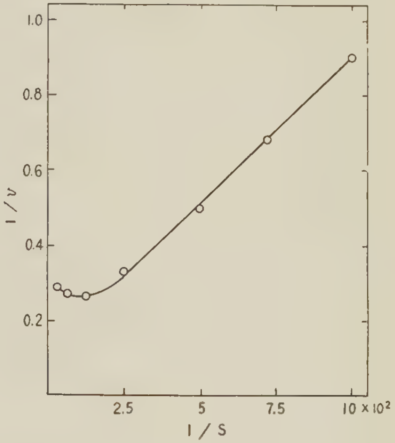

Fig. 4. Effect of DL-homoserine concentration. Ordinate, reciprocal of velocity, where v = CO_2 evolved (μl.) per minute; abscissa, reciprocal of homoserine concentration. ATP concentration was 24 μM per vessel. Other conditions were the same as noted in Fig. 2.

preincubation was made with homoserine, Mg^{++} and Zn^{++} were the activators of the system, but Mn^{++} was slightly inhibitory (Table III, Experiment 1). Mn^{++} and Zn^{++} showed opposite effects in this respect. This fact may offer a clue to the mechanism of metal activation in this system. Detailed experiments and discussion of metal activation will be given in a forthcoming paper.

TABLE III

Effect of Metals

Metal	Experiment 1		Experiment 2	
	Rate	% Activity	Rate	% Activity
None	2.49	100	1.28	100
Mg^{++}	4.23	170	2.02	158
Zn^{++}	4.57	183	0.48	37
Mn^{++}	2.20	88	2.70	211
Co^{++}	2.71	108	—	—
Cu^{++}	0	0	—	—

Metal concentration was 5×10^{-3} *M*. NaF was omitted from the reaction mixtures. In experiment 1, metals were preincubated with homoserine solution for 15 minutes at 37°. Other conditions were as noted in the text. Experiment 2 differed from experiment 1 in that metals were preincubated with the enzyme solution, and ATP concentration was 6 μM per vessel.

TABLE IV

Effect of EDTA and Metals

Addition	Rate	% Activity
None	1.57	100
Mg^{++}	2.86	182
EDTA	0.16	10
EDTA & Mg^{++}	2.52	161
EDTA & Zn^{++}	1.70	109

Concentrations of metals and EDTA were 5×10^{-3} *M* and 8×10^{-4} *M*, respectively.

The enzyme was markedly inhibited by PCMB as well as EDTA, but not affected by monoiodoacetate, cyanide, glutathione and hydroxylamine (Table V). In the previous paper, these compounds were shown as potent inhibotors of the crude enzyme system which catalyses the over-all reaction of threonine biosynthesis from homoserine (*3*). Among these, hydroxylamine was proved to inhibit almost completely the transformation of *O*-phosphohomoserine to threonine, and advantage has been taken of this property in the enzymic preparation of *O*-phosphohomoserine from ATP and homoserine, as will be noted in a separate paper (*7*).

TABLE V

Effect of Inhibitors

Compound	Concentration	% Inhibition*
	*m*M	
NaF	20	0
NH_2OH	10	0 or activate
KCN	10	<5
Monoiodoacetate	10	0 or activate
PCMB	1	97
Glutathione	10	<5
EDTA	10	91

* Average values of two or three experimental series.

NaF was omitted from all reaction systems.

TABLE VI

Optical Specificity

Substrate	Concentration	Rate
	*m*M	
DL-Homoserine	2	1.38
	4	2.53
	8	3.70
L-Homoserine	1	1.27
	2	2.36
	4	3.81

Condition was the same as noted in the text, except that concentrations of ATP and NaF were 24 μM and 100 μM per vessel, respectively.

Specificity Studies—The enzyme was specific for L-homoserine, and D-homoserine was not utilized (Table VI). Together with our previous findings (*3*), the optical specificity of the reaction sequence leading to threonine can be thus designated, L-homoserine→*O*-phospho-L-homoserine→L-threonine. The naturally occurring hydroxyaminoacids, serine and threonine, did not act as substrate or inhibit the phosphorylation of L-homoserine (Table VII).

The enzyme preparation is heterogeneous and is capable of phosphorylating glucose and fructose in the presence of ATP. Unsuccessful attempts were made to separate these activities from homoserine kinase. However, the ratios of the specific activities of homoserine kinase and hexokinase at the initial and the final stages of purification were found to be markedly different. Homoserine kinase was concentrated about 15 to 20-fold by the purification procedure, while hexokinase activity was increased about 1.5 to 2.0-fold with glucose as substrate. Thus the latter enzyme was considered to be an impurity.

TABLE VII

Substrate Specificity

Substrate	Concentration	% Activity
	mM	
DL-Homoserine	12.5	100
DL-Threonine	12.5	<4
DL-Serine	12.5	0
DL-Homoserine & DL-threonine	12.5 each	92
DL-Homoserine & DL-serine	12.5 each	97

All amino acids used were racemic form. Each vessel contained 8 μM of ATP. Other condition was the same as noted in the text, except for the variation of substrate.

SUMMARY

1. A kinase from bakers' yeast which is involved in the biosynthesis of threonine from homoserine has been partially purified with ammonium sulfate fractionation and alumina treatment, and demonstrated to catalyze the reaction, L-homoserine + ATP → O-phosphohomoserine.

2. The enzyme was activated by Mg^{++}, Mn^{++} and Zn^{++}, and markedly inhibited by PCMB and EDTA. This was specific for L-homoserine, and D-homoserine, DL-threonine and DL-serine did not act as substrate. Some other properties were described.

REFERENCES

(*1*) Watanabe, Y., Konishi, S., and Shimura, K., *J. Biochem.*, **42**, 837 (1955)

(*2*) Watanabe, Y., and Shimura, K., *J. Biochem.*, **42**, 283 (1956)
(*3*) Watanabe, Y., and Shimura, K., *J. Biochem.*, **42**, 181 (1955)
(*4*) Watanabe, S., Hyozyun Seikagaku Zikken, Bunkodo, Tokyo, p. 239 (1953)
(*5*) Cohen, S. S., Methods in Enzymology, Academic Press, N. Y., vol., **1**, p. 350 (1955)
(*6*) Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148**, 117 (1943)
(*7*) Watanabe, Y., and Shimura, K., *J. Biochem.* (in press)
(*8*) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism, Burgess Publishing Co., Minn., p. 21 (1951)

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THE ELECTROPHORETIC FRONTAL ANALYSIS OF ENZYMES

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By the Tiselius method of electrophoresis (*1*) it has become possible to study the distribution of proteins in body fluids and organ extracts. The method has been applied also to the enzyme chemistry. But here the method was utilized chiefly to separate enzymes or to test the uniformity of them. On the other hand studies on the distribution of enzymes in biological fluids or in tissue extracts are rare (*2*). This comes from the fact that the fractionation by the boundary method is difficult and not complete, since it is carried out by compensation or by dividing the cell into many compartments (*3*). Moreover, the fractions obtained are usually not simple except those which contain the fastest or the slowest component. According to Tiselius (*4*), the zone electrophoresis has the advantage of separating components materially, not merely optically. So that the study of enzyme distribution in biological fluid becomes possible. Applications of zone electrophoresis, especially paper electrophoresis to the enzyme chemistry are increasing (*5*).

The filter paper technique, however, has certain limitations (*6*). First, the capacity is low, second, adsorption occurs often, and third, elution of material is not easy. The best method would be one without supporting material. Hence in place of the zone electrophoretic method, the authors have made an attempt to analyse enzymatically the fractions forced out dropwise from the electrophoresis cell of moving boundary type. Thus it is a sort of frontal analysis (*7*), as can be seen in the following.

EXPERIMENTAL

Principle—The principle of the electrophoretic frontal analysis is the same as devised by Tiselius and his collaborators for the chromatography (*7*). Only the separation of proteins are effected by the moving boundary electrophoresis, instead of the adsorption chromatography. After the boundary pattern by the usual Tiselius method is obtained, the solutions in each channel of cell are forced dropwise through a micro-conductivity cell. The collected drops are then analysed of their enzymatic activities. The conductivities of the drops are measured in order to correlate the optical and the activity patterns, based on the conformity of the optical and the conductometric patterns (*8*).

Apparatus and Procedure—At first the normal glass cells were used, with the attachments to force the solution and to measure the conductivity, according to Svensson (*8*). The Svensson cell has a connecting piece between the top and the center pieces, into which two glass capillaries with conductivity cells are inserted. After the electrophoresis boundaries are formed in the usual manner, original solution is introduced from the capillary attached to the bottom piece to force the solution through the connecting piece and each capillary with the conductivity cell. The total assembly is somewhat complicated by the three delicate glass capillaries, so that the manipulation is not simple especially in forming the initial boundaries.

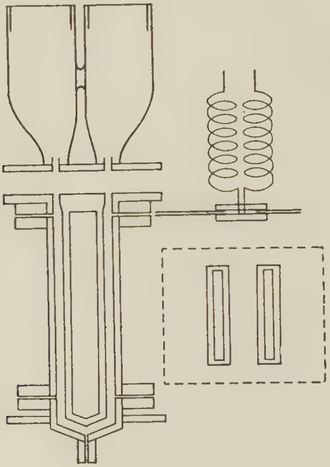


FIG. 1. Electrophoretic cell for frontal analysis of enzyme. On the right side, the cross section of channels is shown.

Afterwards special cells are built for the purpose of the present work according to the ideas of Svensson (*9*) and Brewer *et al.* (*10*) with " Acrylite " (polymetacryl ester) plate. The moving boundary and conductivity cell assembly is shown diagrammatically in Fig. 1. The center and the bottom section of the cell is one piece and not separable. There are provided five capillary holes to be inserted with capillary tubes of polyvinyl ester, of which two upper ones are connected to micro-conductivity cells.

The conductivity cells are built in " Acrylite " block (3×7×22 mm.). The electrodes consist of platinum wires sealed through the wall of the capillary hole (diameter

1.5 mm.) bored in the block. The distance between two electrodes is 2.5 mm. Each cell has a constant of about 6 cm^{-1}.

Filling of the cell is carried out according to Brewer *et al.* (*10*). In order to facilitate the procedure, a three way cock of " Acrylite " is provided at the end of each capillary tube. In filling the cell, *i.e.* in forming the initial boundaries every part of the cell assembly remains unmoved. First, all the apparatus is filled with a veronal buffer of pH 7.8 and ionic strength 0.1, the electrode vessels are arranged for use and the vessels and the capillary tubes are all closed. Then by means of a syringe the protein solution is introduced from the bottom and the buffer is forced from the lower capillary on the ascending side, then from the upper one on the descending side, until sharp boundaries are made at the level of the holes. Finally the upper capillaries are filled with buffer and the lower ones with protein solution.

After the desired separation of the boundaries is achieved, the solution in each channel is forced through each upper capillary and collected dropwise, by introducing the original solution from the bottom capillary. On the way, the conductivities of the solution are measured.

Material—The bovine sera used are usually diluted with the same volume of a veronal buffer of pH 7.8 and ionic strength 0.1, and 100 ml. thereof are dialyzed against 500 ml. of the buffer in a refrigerator for 48 hours.

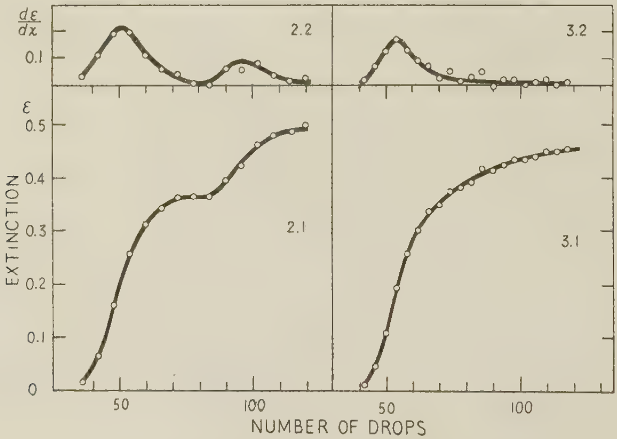
Methods of analysis—Nitrogen is determined by the usual micro-Kjeldahl method and sometimes by the increment of refractive index.

The activity of alkaline phosphatase is measured by the *p*-nitrophenylphosphate method (*11*), applied to the serum by Bessey *et al.* (*12*). To the collected drops of protein solution, 1 ml. of substrate solution is added, after both solutions have been warmed to the temperature, 38°, in the thermostat. The substrate solution consists of 0.5 ml. of 0.4 per cent sodium *p*-nitrophenylphosphate and 0.5 ml. of 0.1 *M* carbonate buffer of pH 9.8, containing 1 m*M* $MgCl_2$ as activator. After the incubation of 30 minutes at 38°, 5 ml. of 0.1 *N* NaOH is added and the extinction at 410 mμ. is determined with a Beckman DU type spectrophotometer.

RESULTS AND DISCUSSION

In Figs. 2 and 3, Curves 2.1 and 3.1 are the representations of the activities of alkaline phosphatase in the ascending and descending channels, respectively. In the run of experiments here described, two drops at a time are used with a interval of four drops on the ascending, and two drops on the descending side, for the measurement of enzyme activity. The activity curve shows two steps or boundaries on the ascending side and only one on the descending side. These boundaries can be visualized as peaks if the changes of activity are plotted against the number of drops. Curves 2.2 and 3.2 show the changes of activity, *i.e.* the differences of two adjacent values of activities. Two peaks of activity on the ascending and one on the descending side are readily seen. It is the same procedure, as in the case of refractive index gradient, provided that the latter is observed immediately instead of calculating the differences.

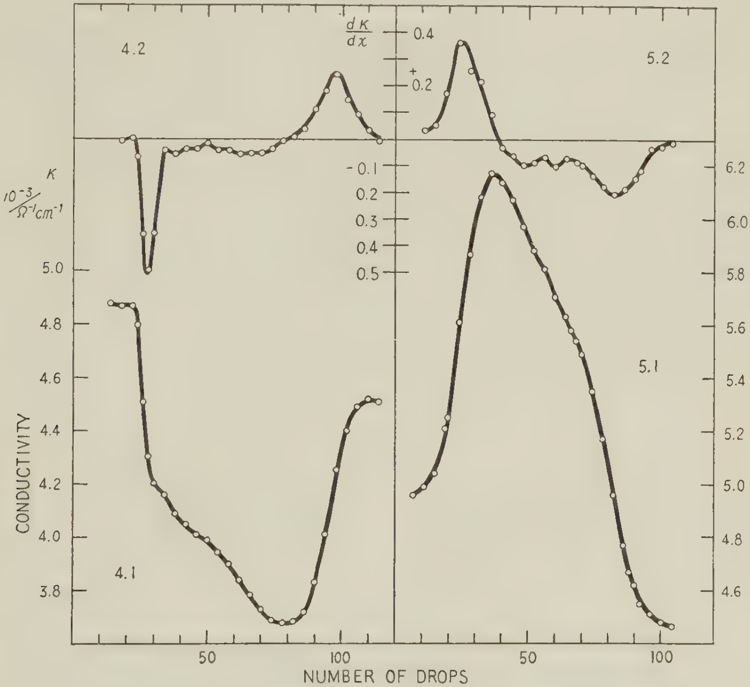
The number of drops correlates to the line of electrophoresis inversely on the ascending side and directly on the descending side, since the drops are forced from the upper holes on both sides. But the correlation between the boundaries of enzyme activity and those observed optically of proteins can not be established immediately, since it is very difficult to correlate directly the number of drops and the optically observed boundaries.



FIGS. 2 and 3. Activity curves of alkaline phosphatase of bovine serum on the ascending, respectively descending sides.
Explanation, see text.

As reported by Svensson (*8*), the patterns obtained by plotting the conductivity gradient correspond to those obtained optically. Thus the number of drops and the optical pattern can be correlated by means of the pattern obtained conductometrically. The measurement of conductivity is carried out in the capillary tube through which the solution is forced. The volume of the tube from the conductivity cell to the opening of the capillary can be measured exactly, so that the coordination of the number of drops with the conductivity gradient curve, therefore with the optical pattern, can be exactly established.

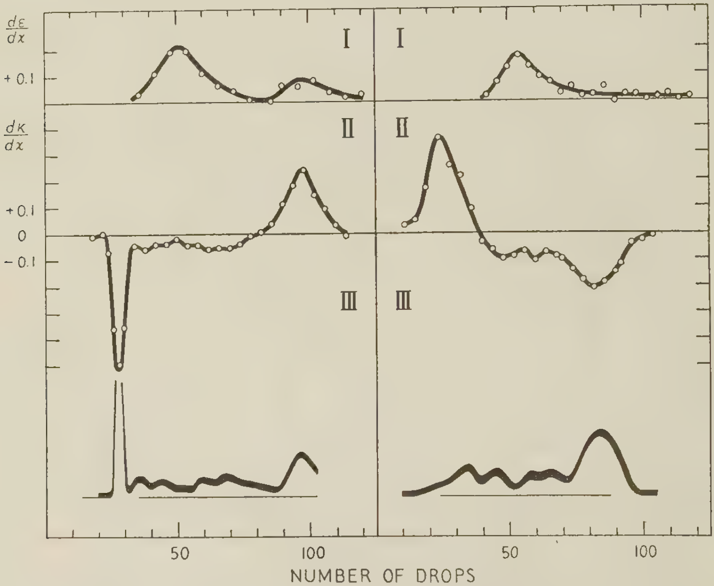
In Figs. 4 and 5, Curves 4.1 and 5.1 show the conductivity of the solutions forced from the ascending and descending channels, respectively. The change of conductivity, *i.e.* the conductivity gradient curve is represented by Curves 4.2 and 5.2. In each conductometric pattern there can be noticed several peaks, which must correspond to those of the optical pattern. The optical pattern can be so plotted that the pair of corresponding peaks on both curves stand on the same number of drops namely on the same position on the line of electrophoresis.



FIGS. 4 and 5. Conductivity and its gradient curves of bovine serum on the ascending, respectively descending sides.
Explanation, see text.

In Figs. 6 and 7, are brought together the gradient pattern of enzyme activity (I), that of conductivity (II) and that of refractive index (III).

As already mentioned, (I) and (II) can be correlated with each other by the number of drops, so that the peak of alkaline phosphatase lies in the 51st drop on the ascending and in the 54th on the descending side. The peaks obtained on (II) and (III) must correspond with each other. Unfortunately the conductometric peaks are not clear enough, except the two remarkable peaks of albumin and anomaly (δ, respectively ε). The coordination, however, of the two patterns, (II) and (III), can be carried out by coordinating these two peaks. Thus it can be observed from the figures that the peak of alkaline phosphatase falls between those of α and β-globulins. The peaks of albumin and anomaly lie on the ascending side in the 28th and 98th drops, respectively, and on the descending side in the 82nd and 22nd drops, respectively. Hence the the mobility of alkaline phosphatase relative to albumin can be calculated on the ascending side to be 67, and on the descending side to be 53.



Figs. 6 and 7. Gradient curves of alkaline phosphatase activity (I), conductivity (II), and refractive index (III) of bovine serum on the ascending (Fig. 6), respectively descending sides (Fig. 7). Explanation, see text.

The pattern of alkaline phosphatase on the ascending side, Curve 6 (I), shows two peaks. As can be seen from the corresponding conductometric pattern (II), the second peak lies on the position of δ-anomaly. From this fact it is very probable that the second peak would be due to the initial concentration difference between serum and buffer solution. It can be supported also from the following fact: The results obtained by Hayashi (*13*) show a more distinct, larger second peak. As the concentration difference was larger in his experiment than in the present one, it can be assumed that the larger the concentration, the larger the second peak. The reason, however, for the absence of the correspondino second peak on the descending side, is to be investigated further.

Finally some remarks must be made on the availability of the method of electrophoretic frontal analysis proposed here. The distribution of an enzyme may be studied by the method, as can be seen from the results already shown. In the method proposed, however, the volume of enzyme solution for one measurement is limited and the number of measurements is fairely large. So that not all enzymes can be investigated by it. The alkaline phosphatase is a very favourable enzyme to test the applicability of the method, since its measurement is micro-chemical and simple. But considering the accuracy of the enzymatic measurement, it would be very difficult to distinguish two enzymes of the same kind and similar mobility, if the activity of one of them were under about 1/10 of the other.

SUMMARY

1. After the electrophoretic boundaries are obtained by the usual Tiselius method, the solutions in ascending and descending channels are forced out dropwise and the activity of the alkaline phosphatase of the drops is measured.

2. Plotting the differences of activity between adjacent drops, a pattern of alkaline phosphatase is obtained.

3. The pattern of phosphatase can be correlated with the optical pattern by means of a differential conductivity curve of the drops.

4. The alkaline phosphatase of bovine serum shows a mobility, which falls between those of α and β-globulin. Its relative mobility in regard to albumin is 67 and 53 on the ascending and descending sides, respectively, at pH 7.8 and ionic strength 0.1.

5. Applicability of the method to other enzymes is also discussed.

REFERENCES

(*1*) Tiselius, A., *Trans. Faraday Soc.*, **33**, 524 (1937) and many others.

(*2*) Byrne, Y. M., Phinney, J. I., Schachter, M., and Young, E. G., *J. Biol. Chem.*, **192**, 683 (1951). Grossberg, L., Komaro, S. A., and Shay, H., *Am. J. Physiol.*, **168**, 269 (1952). Takahashi, M. and Hayashi, Y., *Seibutsu-butsuri-kagaku*, **1**, 97 (1951). Works concerning the test of uniformity and separation of enzymes are not cited.

(*3*) Svensson, H., Advances in Protein Chem., Acad. Press Inc., N. Y., **4**, 251 (1948)
Theorell, H., Handbuch d. biol. Arbeitsmethoden, V, 10, **4**, 1097 (1938)

(*4*) Tiselius, A. and Per Flodin, Advances in Protein Chem., Acad. Press Inc., N. Y., **8**, 461 (1953)

(*5*) Wetter, L. R. and Corrigal, J. J., *Nature*, **174**, 695 (1954). Crestfield, A. M. and Allen, E. W., *J. Biol. Chem.*, **211**, 363 (1954). Toschi, G. and Boccacci, M., *Bull. soc. ital. biol. sper.*, **30**, 563; 565; 567 (1954); Bajusz, E. and Kovary, L., *Plasma*, **2**, 615 (1954); (Cited from C. A.). Roche, J. and Bouchilloux, S., *Bull. soc. chim. biol.*, **35**, 567 (1953). Jerwyn, M. A. and Thomas, R., *Biochem. J.*, **56**, 631 (1953). Norpoth, L., Surmann, T., and Cloeges, J., *Klin. Wochschr.*, **31**, 1005 (1953). And many others.

(*6*) Kunkel, H. G. and Slater, R. J., *Proc. Soc. Expr. Biol. Med.*, **80**, 42 (1952)

(*7*) Tiselius, A., Advances in Protein Chem., Acad. Press Inc., N. Y., **3**, 67 (1947)

(*8*) Svensson, H., *J. Am. Chem. Soc.*, **72**, 1974 (1950)

(*9*) Ibid., *Arkiv Kemi. Mineral. Geol.*, **22A**, No. 10 (1946)

(*10*) Brewer, E. and Dreblow, E. S., *Biochem. J.*, **49**, 253 (1951)

(*11*) Ohmori, Y., *Enzymologia*, **4**, 217 (1937)

(*12*) Bessey, O. A., Lowry, O. H., and Block, M. J., *J. Biol. Chem.*, **164**, 321 (1946)

(*13*) Hayashi, Y., *Bull. Yamaguchi Med. School*, in press.

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CONTRACTILE PROTEINS FROM ADDUCTORS OF PECTEN

III. FAILURE TO DETECT ADENYLIC ACID DEAMINASE ACTIVITY

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Since Schmidt (*1*) demonstrated the presence, in rabbit muscle, of an enzyme which was specific in deaminating adenylic acid (AMP), many investigations have been done on the subject. Recently, several authors (*2-4*), working with the rabbit striated muscle, ascribed the observed AMP deaminase activity to the contractile protein in the muscle. Perry (*5*) and Green *et al.* (*6*) showed that the addition of adenosine triphosphate (ATP) to their respective myofibril and myosin gave rise to the production of a significant amount of inosinic acid (IMP) as the result of successive actions of adenosinetriphosphatase, adenylate kinase and AMP deaminase. Some of these authors are in line in claiming the identity or a very close association of this enzyme to myosin or actomyosin (see *4*).

In remarkable contrast to these facts, we have previously demonstrated that myosin B from pecten adductor muscle was practically devoid of the capacity of producing any appreciable amount of IMP from added ATP, in spite of the presence (as a contaminant in the myosin B preparations) of adenylate kinase, actually converting part of the added ATP into AMP (*7*). In the present study, AMP deaminase activity of the muscle homogenate and myosin B from fast-striated adductor of pecten (*P. yessoensis*) were tested in the presence of added AMP. The results thus obtained were in full accordance with our previous finding, as will be described in more detail in the following.

EXPERIMENTAL

Muscle homogenate and myosin B were prepared from pecten fast adductor as previously described (*7*). One ml. of the homogenate contained 0.5 g. portion of fresh muscle. AMP was prepared according to the method of Kerr (*8*) and purified through a column of Dowex 1 resin. All the experiments in this study were carried out at 36° and for varying lengths of incubation time (0–60 minutes), pH of the reaction medium, 5.0–8.5. 0.05 *M* succinate buffer and 0.05 *M* borate buffer were used, respectively, below and above pH 7.0. Divalent cations were not added, because their effects were not very marked (*9*). Deamination of the substrate was followed by the direct Nesslerization of the filtrate solution (Vanselow's reagent (*10*), pH of the reaction mixture below 7.0), or by measuring the change in optical densities at 265 mμ and 240 mμ (*11*). The content of protein was calculated by multiplying a factor of 6 to the nitrogen content determined by the micro Kjeldahl method. The pH was determined with a Beckman type G pH-meter. Optical density was measured with a Shimazu ultraviolet spectrophotometer.

TABLE I

The Absence of AMP Deaminase Activity of Pecten Adductor Muscle

One ml. of incubation mixture contained 0.6 *M* KCl, 1 mM AMP and 1.2 mg. of myosin B (or 0.2 ml. of the homogenate). Two ml. of the mixture was withdrawn at definite time intervals and the reaction was stopped by mixing with 0.5 ml. of 18 *N* sulfuric acid. The figures are amounts of IMP produced during 60 minutes, and these values are hardly beyond the limits of experimental error.

pH	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
IMP (*mM*)	<0.02	<0.02	<0.02	<0.03	<0.03	<0.01	<0.01	<0.01
		(<0.05)*			(<0.05)*			

* The figures in parenthesis are the values for the homogenate.

RESULTS AND DISCUSSION

In Table I are summarized the results obtained on the deaminase activity of myosin B and the homogenate of pecten fast adductor muscle. Under the various experimental conditions studied, no significant production of ammonia or conversion of AMP to IMP was ever detected. This result is striking contrast to the high deaminase activity of myosin and actomyosin from rabbit skeletal muscle.

There are some reports, although not many so far as we know, mentioning the absence of AMP deaminase activity in striated muscles or contractile protein prepared therefrom. Gilmour and Calaby (*12*) obtained negative results with the locust striated muscle and its actomyosin preparation. Anina (*13*), working with crayfish, arrived at the same conclusion. It is to be noticed that all these negative results as regards the AMP deaminase activity were obtained with invertebrates and all the instances connected with high AMP deaminase activity, on the other hand, were limited to striated muscle of vertebrate. On a homogenate of claw muscle from crab (an invertebrate), Conway and Cooke (*14*) showed a very low production of ammonia from AMP (4 per cent of rabbit skeletal muscle at the same condition), under extremely high substrate concentration (1–2 per cent). However, there might be other pathways to produce ammonia from AMP such as the successive actions of 5′-nucleotidase and adenosinedeaminase in the used homogenate. Anyhow the obvious difference from rabbit should be noticed in this case too.

Therefore, it seems likely that here we are dealing with another biochemical feature probably discriminating between vertebrate and invertebrate muscle in addition to the well known difference in the nature of phosphagens and guanidine kinases.

SUMMARY

1. The adenylic acid deaminase activity of the homogenate and myosin B of pecten fast adductor was estimated to be negligibly small.

2. Some comparative aspects have been discussed on the adenylic acid deaminase.

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REFERENCES

(*1*) Schmidt, G., *Z. physiol. Chem.*, **179**, 243 (1928)
(*2*) Engelhardt, V. A., Comm. au 2ième Congr. Intern. de Biochim., Paris, p. 50 (1952)
(*3*) Ferdman, D. L., and Nachiporenko, S., *Ukrain. Biochem. J.*, **18**, 105 (1946)
(*4*) Hermann, V. S., and Josepovits, G., *Nature*, **164**, 845 (1949)
(*5*) Perry, S. V., *Symp. Soc. Exptl. Biol.*, **9**, 203 (1955)
(*6*) Green, I., Brown, J.R.C., and Mommaerts, W.F.H.M., *J. Biol. Chem.*, **205**, 493 (1953)
(*7*) Tonomura, Y., Yagi, K., and Matsumiya, H., *Arch. Biochem. and Biophys.*, **59**, 76 (1955); *ibid.*, **64**, 466 (1956)
(*8*) Kerr, S. E., *J. Biol. Chem.*, **139**, 131 (1941)

(*9*) Humphrey, G. F., and Webster, H. L., *Austral. J. Exptl. Biol., Med.*, **29**, 17 (1951)
(*10*) Vanselow, A. P., *Ind. Eng. Chem.* (*Anal. Ed.*), **12**, 516 (1940)
(*11*) Kalckar, H. M., *J. Biol. Chem.*, **167**, 429 (1947)
(*12*) Gilmour, D., and Calaby, J. H., *Enzymologia*, **16**, 23 (1953)
(*13*) Anina, I. A., *Ukrain. Biochem., J.*, **27**, 137 (1955)
(*14*) Conway, E. J., and Cooke, R., *Biochem., J.*, **33**, 479 (1939)

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HYDRAZINOLYSIS OF YEAST RIBONUCLEIC ACID. FORMATION OF " RIBO-APYRIMIDINIC ACID "

Sirs:

It is well known that mild acid hydrolysis of DNA, but not of RNA, is followed by the formation of " apurinic acid " owing to the cleavage of purine deoxyribosidic bond. However " apyrimidinic acids," nucleic acids derivatives devoid of pyrimidine bases, have not yet been obtained. Such compounds, if obtained, may be useful for the elucidation of the chemical structure of nucleic acids.

It was shown that hydrazine reacted on uracil to form pyrazolone and urea (*1*), and on uridine to form pyrazolone and, presumably, ribosylurea (*2*). Further studies on the action of hydrazine hydrate upon pyrimidine ribonucleosides and ribonucleotides have been carried out in detail by Baron and Brown (*3*) in 1955, and have led them to the finding of a new excellent chemical method for the determination of the orientation of the phosphoryl group in the pyrimidine nucleotide isomers, a and b. In the case of the nucleotides, uracil and cytosine residues have been decomposed to pyrazolone and 3-aminopyrazole, respectively, and simultaneously, ribose 2- and 3-phosphoric acid have been formed from pyrimidine nucleotide a and b, respectively. We applied the hydrazinolysis to yeast RNA as follows with the expectation to obtain apyrimidinic acid.

Sodium ribonucleate from yeast (1 g.) was treated with anhydrous hydrazine (4 ml.) at 60° for 60 minutes, and after the addition of 4 volume of alcohol, the precipitate was dissolved in 10 ml. of distilled water and dialyzed. From the non-dialyzable fraction, to which alcohol was added untill the concentration of alcohol reached to 20 per cent, a product was precipitated on cautious addition of HCl to pH 2. The product thus obtained in considerable yield (0.73 g.) was less soluble at neutral pH than RNA.

(*1*) *Nitrogen and Phosphorus Content*—As shown in Table I, the hydrazinolysis of RNA resulted in the formation of the product of which the nitrogen content and the N/P ratio were lower than those of the original RNA. These results seem to mean a loss of some parts of nitrogenous components from the original RNA, and it may be supported by the data described below and also by the previous investigations by others that the components which are lost are the pyrimidine bases.

(*2*) *Orcinol Reaction*—As shown in Table II, the product gives a far stronger orcinol reaction than the original RNA. It may be explained by the removal of pyrimidine bases in the hydrazinolysis.

TABLE I

Analyses of the Non-dialyzable Product Obtained by the Hydrazinolysis of Yeast RNA—Part 1

	N	P	N/P	atomic N/P
Yeast RNA sodium salt, purified*	14.5%	8.4%	1.73	3.8
Yeast RNA, theoretical**	16.7	9.6	1.74	3.9
Yeast RNA, pyrimidine-less, theoretical**	13.7	11.0	1.25	2.8
Non-dialyzable product from hydrazine treated yeast RNA	12.4	1.36	1.36	3.0

* The preparation of Schwarz Lab. Inc. was purified by chloroform-gel.

** Theoretical value for a large polynucleotide molecule consisting of tetranucleotides containing 1 mole each of adenine and uracil, 1.2 moles of guanine, and 0.8 mole of cytosine.

TABLE II

Analyses of the Non-dialyzable Product Obtained by the Hydrazinolysis of Yeast RNA—Part 2

	Reducing power of ribose	RNA-phosphorus by Bial reaction	Ultraviolet absorption	
			Maximum	$\varepsilon_{max}(P)$
Yeast RNA sodium salt, purified*	0.0%	(8.4)%	259 $m\mu$	8,950
Yeast RNA, pyrimidine-less, theoretical**	24.0	16.6	—	—
Non-dialyzable product from hydrazine treated yeast RNA	19.6	15.0	255.5	6,600

* See foot note*, Table I.

** See foot note**, Table I.

(*3*) *Reducing Power*—The estimation of reducing power of the product is a measure of ribosidic linkage in the RNA, the cleavage of which cause the appearance of reducing group. According to the estimation of reducing sugars by means of Fujita-Iwatake's method (*4*) which is a modification of Hagedorn-Jensen's, the product proved to be in marked contrast to the original RNA ; the reducing power of the latter was none, but that of the former was about 20 per cent of that of the ribose, which corresponded to the reducing ribose residues of about 37 per cent of the total ribose residues in the former (Table II). Another evidence for the existence of the reducing sugar group in the product was given from the colour development of the product on filter paper by the spray of aniline hydrogen phthalate reagent for reducing sugars.

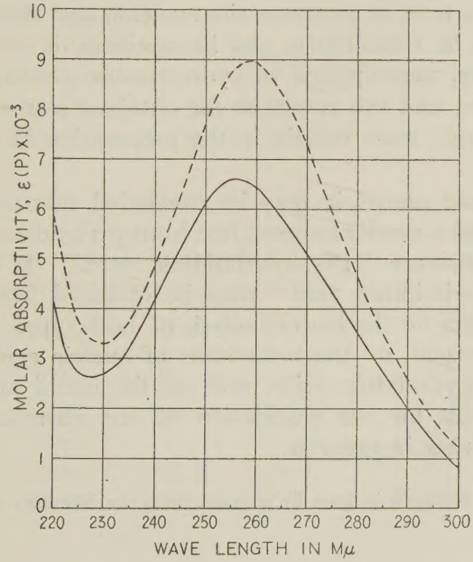

FIG. 1 Ultraviolet absorption curves of yeast RNA (brokn line) and its hydrazine treated product (solid line) in phosphate buffer of pH 7.0.

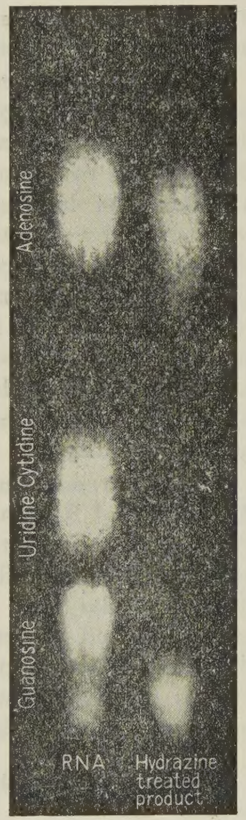

FIG. 2 Paper chromatogram of nucleosides liberated from yeast RNA and its hydrazine treated product on hydrolysis with lanthanum hydroxide gel at 80°, pH 8, for 40 hours.

Solvent system: 86 per cent (*v*/*v*) aq. *n*-butanol, with 5 per cent by volume of concentrated NH_3 solution, descending.

(*4*) *Ultraviolet Absorption*—RNA solution shows a maximum absorption of ultraviolet light at the wavelength of 258–260 mμ in neutral pH range. The product showed a maximum absorption at 255.5 mμ. The shift of the position of the maximum absorption towards shorter wavelengths by the hydrazinolysis may be also explained by the removal of pyrimidine bases. The extinction coefficient based on one gram-atom of phosphorus per liter, $\varepsilon(P)$, was much lower than that of RNA. The ratio of $\varepsilon(P)$ of the reaction product to that of RNA was about 0.74.

(*5*) *Detection of Bases*—RNA has been known to give purine and pyrimidine nucleosides and inorganic phosphate almost quantitatively on hydrolysis with lanthanum hydroxide gel at pH 8, 80° (*5*), for about 40 hours. The hydrazinolysis product was submitted in the same way to the action of lanthanum hydroxide, and the liberated nucleosides were identified by chromatography on filter paper. None of pyrimidine nucleosides were detected on the chromatogram (Fig. 2). To obtain further confirmations, enzymes such as pancreas ribonuclease and ribonuclease T_1, which was found in Takadiastase and its specificity is now being studied in our laboratory, were applied to hydrolyze the product of hydrazinolysis to nucleotides, and the results so far obtained seemed to show that pyrimidines, if any, must remain in the preparation in a very small amount.

From the results mentioned above, it may be concluded that the hydrazinolysis of RNA afforded a product almost free from pyrimidines. We should like to call the substance " ribo-apyrimidinic acid." It is expected that " deoxyribo-apyrimidinic acid " may be obtained from DNA in a similar way. Studies on the hydrazinolysis of both types of nucleic acids and of apurinic acid, on the behaviours of nucleic acids hydrolyzing enzymes upon apyrimidinic acids, and on the useful applications of apyrimidinic acids for the elucidation of the chemical structure of nucleic acids are now in progress.

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REFERENCES

(*1*) Fosse, R., Hieulle, A., and Bass, L. W., *Compt. rend.*, **178**, 811 (1924)
(*2*) Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **71**, 167 (1926)
(*3*) Baron, F., and Brown, D. M., *J. Chem. Soc.*, 2855 (1955)
(*4*) Fujita, A., and Iwatake, D., *Biochem. Z.*, **242**, 43 (1932)

(*5*) Shimomura, M., and Egami, F., *Bull. Chem. Soc. Japan*, **26**, 263 (1953): Shimomura, M., *ibid.*, **27**, 29 (1954)

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